Report _____

CELLULAR AND MOLECULAR LEVEL RESPONSES AFTER RADIOFREQUENCY RADIATION EXPOSURE, ALONE OR IN COMBINATION WITH X-RAYS OR CHEMICALS

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Dr. Walter J. Kozumbo
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Dear Dr. Kozumbo:

Enclosed please find the original and three copies of the final report for our Air Force Office of Scientific Research Project No. AFOSR-91-0206.

Because of a verbal commitment I made to Marilyn J. McKee, Chief, Contracts/Grants, Administration Division, AFOSR, to submit the final report on this date, I have submitted it without finalizing the required Report Documentation Page. I will obtain certain information that is missing information from you as soon as possible, and immediately submit a final corrected copy of the Report Documentation Page.

Please accept my apology for proceeding in this time order; however, because my tardiness has resulted in our organization being placed on the Delinquent Awardee List, and since my pending grant award was thereby jeopardized, I felt compelled to proceed as expeditiously as possible.

Sincerely,

Martin L. Meltz, Ph.D.
Principal Investigator

MLM/pr

TABLE OF CONTENTS

List of Figures

Appearing in Appendix

List of Tables

Appearing in Appendix

INTRODUCTION

The focus of this research was oriented towards the effects of physical agents, in particular non-ionizing (microwave) and ionizing radiation, on the growth, viability, genetic effects and metabolic activation in a representative human cell line. While an initial area of interest was the primary human lymphocyte, consultation with scientists well versed in using such cells revealed a concern that subtle changes due to radiation exposures would be masked by the variability inherent in the use of primary blood isolates. During the early part of this project, as summarized in the annual reports previously submitted, attention therefore focused on the use of an Epstein-Barr Virus (EBV) transformed human lymphoblastoid cell line. This cell line, the 244B cell, is derived from B cell origin. To enhance reproducibility, the growth pattern of the cell was carefully determined and specific culture conditions decided upon. Additionally, careful attention was given to always initiating mass cultures for experimental purposes at the same cell density, and collecting the cells at the same time after initiation and at the same cell density (during a period of rapid exponential growth). The response of the cells to different doses of ionizing radiation, with viability measured by dye exclusion and cell cycle distribution analyzed using flow cytometry at different times after exposure, has been described in previous reports. The dose range examined in those studies, for ionizing radiation exposure, was very extensive, with some of the doses achieved being in the lethal range (up to 10 Gy), and incubation times after irradiation extending out to 120 hrs.

All of these studies were in preparation for undertaking examination of potential interactions between ionizing radiation and microwave radiation exposures. As our research progressed beyond the previously described characterizations, two very important decisions were made. Because we had planned to undertake low dose and low dose-rate studies with ionizing radiation, alone and in combination with microwave radiation exposures, and because of operational difficulties with the very old Maxitron Orthovoltage X-ray unit in our unique anechoic chamber exposure facility, the decision was

made to examine the effect of low dose gamma ray radiation in the desired low dose range (10 - 200 cGy), but at a standard dose rate emitted from our ^{137}cesium gamma ray source (located in an adjacent room). Also, because there was evidence in the literature that NF-*k*B was an important biochemical factor involved in controlling metabolic activity in mammalian cells, and because of interest in the radiofrequency and extremely low frequency scientific community that exposure in these frequency ranges might induce immediate early gene expression, we turned our focus to this area.

In pursuing this line of investigation, we were aware that studies with mRNA would require large numbers of cells, while our custom-designed flask-holder for exposures in the microwave field was limited to four T-25 flasks. Instead of presuming that cells could be irradiated at high cell densities without altering subsequent results, as often has been done in the past with ionizing radiation exposures, it was decided to carefully examine the impact of incubating and treating cells at various cell densities with a chemical inducer of the nucelar factor *k*B (NF-*k*B). The results of these studies, extensively described in our published manuscript 1 (see Appendix), became very important to our work and to anyone investigating stress responses in mammalian cells.

Once the experimental handling of cells was carefully defined, a series of experiments was undertaken with low dose ionizing radiation, in a dose range which previously had not been explored relative to NF-κB induction, and where one could expect the irradiated cells to remain predominantly viable. A high viability would be expected also after the microwave exposures being considered (where heating was not involved). This reseach direction, while not involving microwave radiation, is in the forefront of the scientific community in looking at the low dose induction of DNA binding proteins and immediate early gene synthesis. This information is contained in the published, being revised and about-to-be submitted manuscripts in the appendix of this report.

We also were aware of the interest, predominantly in the extremely low frequency ELF community but also in the radiofrequency radiation (RFR)

community, of reported ELF effects on melatonin synthesis. Melatonin is an important human hormone synthesized by the pineal gland, and Dr. Russel Reiter, an expert in this area, had described the melatonin (personal communication) as a potent hydroxyl radical scavenger. This allowed him to suggest the hypothesis that a decrease in melatonin due to ELF exposure might be of importance with respect to cancer. The combined backgrounds of the research team of this project in ionizing radiobiology led us to propose the hypothesis that (if the hydroxide radical scavenger concept was correct), melatonin could be expected to serve as a radioprotector. Dr. Vijayalaxmi had become a member of the research staff of this project after its initiation, providing a unique opportunity to examine this hypothesis with freshly isolated human lymphocytes. Approval was obtained from the Human Use Institutional Review Board, and experiments were performed to investigate this hypothesis. If the melatonin was not a radioprotector against ionizing radiation induced DNA damage, as measured by examination of chromosome aberration induction, then any changes described showing reduction in melatonin after ELF exposures would be of much less interest, and probably not of value for study with respect to RFR exposure. The results of these studies, with one manuscript published and one revised and resubmitted (the latter examined micronuclei induction, as compared to chromosome aberration induction), are summarized in the results section and appear in the **Appendix**.

During this project period the development of the Automated Thermal Control System was completed. This system is to be used to maintain the temperature of the cell culture medium in the T-25 flasks during the microwave exposure. It was first developed and patented by the U.S. Air Force Armstrong Laboratory. Our modifications were extensive; a Continuation in Part of the Patent is therefore being prepared for submission under the auspices of the University of Texas Health Science Center at San Antonio. In addition, the software for the system was completely and independently rewritten in the "C" computer language; this is being submitted for copyright protection by the

university. The documentation for both the Automated Thermal Control System and the associated software is being submitted under separate cover.

SUMMARY OF RESULTS

STUDIES ON THE INDUCTION OF NF-κB AND IMMEDIATE EARLY GENES BY LOW-DOSE IONIZING RADIATION

Effects of High Cell Density Stress on the Expression of Transcription Factor NF-κB.

EBV transformed 244B cells (1 x 10^6) were cultured in RPMI-1640 medium containing 2 mM L-glutamine and 10 mM HEPES and supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin. Effect of high cell density stress on the induction of NF-κB was measured by electrophoretic mobility shift assay (EMSA) after treatment of the cells with PMA at three cell densities (1,2, and 5 x 10^6 cells/ml), and harvesting at various time points (including 1, 8, 16, 36 and 72h). An aliquot of the nuclear extracts prepared for EMSA, after harvesting the cells at 8h, was used to determine the subunit expression of NF-κB at the same three cell densities. Western blot analysis was performed using p50 and p65 polyclonal antibodies. The quantitative measurements were analyzed using a desk top digital imaging method. The PMA treated samples were used as positive controls in all of these experiments. The detailed methodology is given in the published manuscript (see **Appendix**, Manuscript #1).

We have demonstrated, for the first time, that the activation of nuclear factor κB is modulated by cell density. When human lymphoblastoid cells where treated with phorbol 12-myristate 13-acetate (PMA) at a high cell density, a severe inhibition of expression of NF-κB resulted. When the cell density during PMA induction was 1 x 10^6 cells/ml, the expression was maximum at 8h; the expression at this time was less at the higher cell densities of 2.5 and 5.0 x 10^6 cells/ml. The expression was also dependent on the time at which the cells were harvested, with higher levels being observed a second time at 36h, following negligible expression for all three cell densities at 16h. It was also observed

that high cell density stress interferes with the nuclear localization of NF-κB subunits (p50/p65). NF-κB subunit p50 expression was highest at the lowest cell density, while p65 expression was unaltered at all three cell densities. (See **Appendix**, Manuscript #1: Toxic Substance Journal 13:159-170).

Activation of Nuclear Factor κB in Human Lymphoblastoid Cells by Low-dose Ionizing Radiation.

EBV transformed 244B cells (1 x 10^6) were cultured in RPMI-1640 medium containing 2 mM L-glutamine and 10 mM HEPES and supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin. The dose (0.25 - 2.0 Gy) and time dependent (1, 8, 16, 36 and 72h) activation of NF-κB after ionizing radiation exposure was measured by EMSA analysis. An aliquot of the nuclear extracts from cells harvested at 8h and prepared for EMSA was used to determine the subunit expression of NF-κB in the same dose range. The quanti-tative measurements were analyzed using a desk top digital imaging method. Mock irradiated and PMA treated samples were used as negative and positive controls, respectively. The detailed methodology is given in the published manuscript (see **Appendix**, Manuscript #2).

We have demonstrated that the exposure of a human lymphoblas-toid cell line to low-dose ionizing radiation (0.25 - 2.0 Gy), where cell viability remains high, will cause a transient increase in the DNA binding activity of NF-κB with increasing incubation time after exposure. The increase was maximal after 0.5 Gy. The measured activation was lower when the radiation dose was raised to 1.0 and 2.0 Gy. To our knowledge, these data are the first demonstration of NF-κB DNA binding activity after such low doses. It was also demonstrated that the activation of NF-κB after low dose ionizing radiation is time-dependent. This study revealed that the activation of NF-κB after 0.5 Gy reached its maximum at 8h, was lower by 16h, had increased again at 24h and reached a second maximum at 36h; it was at this same level at 72h. The maximum activation of NF-κB observed, for any given time post-exposure and all doses studied, was at 8h after the dose

of 0.5 Gy. This biphasic nature of NF-κB activation over time remains unexamined and unexplained.

This study also showed for the first time the differential regulation of NF-κB subunit protein levels after low dose ionizing radiation exposure. The expression of both subunits (p50 and p65) was maximum after 0.5 Gy, compared to the doses of 0.25, 1.0 or 2.0 Gy. The finding that the presence of the heterodimer (p50/p65) in the nucleus was greatest after 0.5 Gy exposure is consistent with the electrophoretic mobility shift assay (EMSA) results. This differential regulation of NF-κB subunits by low-dose ionizing radiation could either be due to the differential activation of pre-existing protein and/or differential induction at the level of gene expression (mRNA synthesis) of the subunit precursors of NF-κB. Although several other studies have documented an increase in NF-κB DNA binding activity following irradiation, the finding that this effect can occur at such low doses and that the subunits are differentially induced by this stimulus is of considerable interest. Further, it was observed that the induction of NF-κB by low dose ionizing radiation followed a different time course of induction than that reported in the literature after high-dose exposures. The maximum expression of NF-κB after low-dose ionizing radiation occurred at 8h after exposure, compared to the 2-4h reported after high-dose exposure [e.g., Brach, et al., 1991]. Of possible importance is that the time frame observed after low-dose ionizing radiation is similar to that reported in UV irradiated HeLa Tk^- cells [Stein, et al., 1989] or PMA treated primary human T cells [Granelli-Piperno and Nolan, 1991].

These findings allow for several possible explanations; that (i) different mechanisms are involved after exposure to different dose levels of ionizing radiation and that this induction could be associated with different biological outcomes, (ii) the shift in time at which a maximum induction of NF-κB is observed could depend on the cell type, or (iii) mechanistically, different signaling pathways may be involved in the different dose ranges. With regard to biological outcomes, the activation of NF-κB after exposure to low-dose ionizing radiation could be a rapid response serving as a protective measure

against radiation insult or injury to cells, and/or a communication pathway to other cells. Moreover, the activation of NF-κB after low-dose ionizing radiation could potentially regulate the expression of genes that code for many gene products supportive of cell survival, thereby circumventing long-term cellular damage, while after exposure to high doses of ionizing radiation, the response may simply reflect too much damage, and physiological breakdown. (See **Appendix**, Manuscript #2: Radiation Research 138:367-372).

Induction of Nuclear Factor κB after Low Dose Ionizing Radiation Involves a Reactive oxygen Intermediates Signaling Pathway.

EBV transformed 244B cells (1 x 10^6) were cultured in RPMI-1640 medium containing 2 mM L-glutamine and 10 mM HEPES and supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin. The effect of low dose ionizing radiation exposure on the viability of the 244B cells was measured using the Trypan blue dye exclusion method. The dose (0.1 - 2.0 Gy) and time dependent (2, 4, 8 and 24h) activation of NF-κB after ionizing radiation exposure was measured by EMSA analysis. The involvement of the reactive oxygen intermediates signaling pathway in the induction by low dose ionizing radiation of NF-κB was examined by pretreating the cells with an antioxidant, N-acetyl-L-cysteine (NAC). The results were compared with those occurring after treatment of the cells with the known free radical inducer, hydrogen peroxide. Quantitative analysis was carried out using a Betascope 603 blot analyzer. Mock irradiated and PMA treated samples were used as negative and positive controls, respectively. The detailed methodology is given in the published manuscript (see **Appendix**, Manuscript #3).

Reactive oxygen intermediates (ROIs) have been reported in the literature to be messengers in the activation of NF-κB in mitogen or cytokine stimulated cells, operating in conjugation with or independently of various other kinase dependent cytoplasmic signaling pathways. Since ionizing radiation is known to generate free radicals in cells, the involvement of the ROI signaling pathway in the induction of NF-κB binding activity after low dose ionizing radiation exposure was examined. The pathway was compared to that occurring in cells

treated with phorbol 12-myristate 13-acetate (PMA) or hydrogen peroxide (H_2O_2). The results not only confirm a previous observation from our laboratory that low-dose ionizing radiation (0.1 - 2.0 Gy) activates κB transcription factor transiently with a maximal induction after a 0.5 Gy exposure, but also demonstrated mechanistically that the activation of NF-κB by low-dose ionizing radiation can be inhibited to a considerable extent by the antioxidant NAC. A 1h pretreatment of 244B cells with 30 mM NAC inhibited more than 55% of the activation of NF-κB induced by a 0.5 Gy exposure, indicating that at least a major part of the activation process is mediated by ROIs. These findings support the previously reported concept that ROIs can regulate the κB element, which in turn can serve as a response element for oxidant stress. (See **Appendix**, Manuscript #3: Radiation Research 140:97-104).

Induction of "Immediate Early Genes" by Low-dose Ionizing Radiation.

EBV transformed 244B cells (1 x 10^6) were cultured in RPMI-1640 medium containing 2 mM L-glutamine and 10 mM HEPES and supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin. The time course of radiation-induced mRNA expression for the protooncogenes c-*fos*, c-*myc*, c-*jun* and c-*Ha-ras* was measured by Northern blot analysis after 0.5 Gy exposures and cell harvesting at 0.25, 0.5, 1, 2, 4, 8, 12 and 16h. The dose response of mRNA expression of these protooncogenes were measured at 1 h after 0.25, 0.5, 0.75, 1 and 2 Gy exposures. To determine the involvement of various second messenger signaling pathways in the induction by low dose ionizing radiation of protooncogenes, the cells were pre-treated with specific second messenger inhibitors for 30 min prior to irradiation and harvested for RNA extraction at 1h postexposure. Mock irradiated and PMA treated samples were used as negative and positive controls, respectively. The detailed methodology is given in the submitted manuscript in revision (see **Appendix**, Manuscript #4).

The *c-fos*, *c-jun*, *c-myc* and *c-Ha-ras* are protooncogenes. These genes on activation are involved in and at least partially responsible for cell proliferation, differentiation, transformation and apoptosis. In this study it was

observed that low-dose ionizing radiation exposure of EBV transformed human lymphoblastoid 244B cells, in the range of 0.25 - 2.0 Gy, differentially induces the protooncogenes *c-fos, c-jun, c-myc* and *c-Ha-ras*. Maximum induction response was seen at 1h after 0.5 Gy for all but c-fos, which showed a maximum response after 0.25 Gy. The time course studies demonstrated that for all four protooncogenes studied, the induction was transient, reaching a maximum at 1h and declining to the constitutive level at 4h post-irradiation. Using various second messenger signaling inhibitors, an initial investigation of the pathways involved was undertaken. All four of the protooncogenes induced by low dose (0.5 Gy) radiation shared a common pathway of tyrosine kinase activation; this was similar to the pathway associated with PMA induction in these cells. Other signaling events are also involved, but these have been shown in this study, using selected inhibitors, to be specific to the protooncogene being activated. Differences in the induction pathways for three of the four protooncogenes have also been observed when 0.5 Gy radiation is the inducing agent, as compared to PMA induction. This study suggests that the cellular response to any single etiological agent, including ionizing radiation, involves a multiplicity of gene activations as well as multiple and different signaling pathways. (See **Appendix**, Manuscript #4: Revised and Resubmitted to Radiation Research).

STUDIES ON THE ABILITY OF MELATONIN TO PROTECT PERIPHERAL HUMAN LYMPHOCYTES AGAINST RADIATION INDUCED CHROMOSOME DAMAGE

Two investigations were conducted using melatonin; in both, human peripheral blood samples were treated with melatonin in vitro, and then exposed to gamma radiation in vitro. The purpose was to determine if pretreatment of the lymphocytes with melatonin decreases the incidence of ionizing radiation-induced genetic damage (chromosome aberrations [study 1] and micronuclei [study 2]), as compared with untreated lymphocytes.

Protection Against Chromosome Aberration Induction

Six separate experiments were conducted at two different times, each using a blood sample from a different donor. The blood donors, 3 males and 3 females, were healthy and non-smoking volunteers aged between 30 and 50 years. Peripheral blood was collected in sterile heparinized vacutainer tubes. From each sample, separate cultures were set up by mixing 0.8 ml whole blood with 9.2 ml pre-warmed RPMI 1640 medium containing 15% fetal bovine serum, 5 U/ml penicillin, 5 μg/ml streptomycin, and 2 mM glutamine. Equal volumes of the freshly prepared melatonin solutions (dissolved in absolute ethanol) were added to the cultures to give final concentrations of 0.5, 1.0 and 2.0 mM. A known radical scavenger, dimethylsulfoxide (DMSO), was used as a positive control at a final concentration of 1.0 M (Littlefield et al., 1988). The solvent control cultures received the same volume of absolute ethanol (final concentration 1%) as used in melatonin treated cultures. All treatments were carried out at 37±1°C in a humidified atmosphere of 5% CO_2 and 95% air, for 20 min. The cultures were then exposed to gamma radiation at room temperature using a ^{137}Cs GammaCell-40 irradiator (Atomic Energy of Canada Ltd.). They were irradiated with a total dose of 150 cGy, delivered at a dose rate of 112.5 cGy/min.

Immediately after irradiation, all cell suspensions were washed with pre-warmed RPMI 1640 culture medium (as above). Separate cultures were then set up using 9.2 ml complete RPMI 1640 medium supplemented with 1% phytohemagglutinin, 15% fetal bovine serum, 5 U/ml penicillin, 5 μg/ml streptomycin, 2 mM glutamine, and 25 μM bromodeoxyuridine, and incubated in the dark at 37±1°C in a humidified atmosphere of 5% CO_2 and 95% air, for 48h. During the last two hours of incubation, colcemid was added to all cultures at a final concentration of 0.1 μg/ml, to arrest the lymphocytes in mitosis.

Following the 48h incubation, lymphocytes were collected, treated with hypotonic solution (75 mM potassium chloride) and fixed in 3:1 methanol:acetic acid mixture. Fixed cells were dropped onto clean microscopic slides, air dried and stained with the standard fluorescence-plus Giemsa technique. At the completion of the experiments with blood samples from the six different donors,

all slides were coded by an individual other than the scorer, and evaluated for chromosome damage. From each culture, 200 cells in their first mitotic division (as defined by the absence of harlequin staining) were examined.

After different treatments, the overall response of the lymphocytes from the six donors was similar, although the absolute values were slightly different. The expected frequencies of abnormal cells showing chromosome damage, exchange aberrations and excess acentric fragments in combined treatment groups, i.e., melatonin+150 cGy, DMSO+150 cGy and solvent control (ethanol)+150 cGy, were computed as the sum of the effects of the two individual treatments minus the frequency in the control (unirradiated and untreated). The statistical significance of the decrease in the observed frequencies relative to the expected ones was evaluated using a one-tailed test [Vijayalaxmi and Burkart, 1989]. Lympho-cytes which were pretreated with melatonin and then exposed to gamma radiation exhibited a significant and melatonin concentration-dependent decrease in the frequencies of abnormal cells, exchange aberrations, and excess acentric fragments, as compared with irradiated lymphocytes which were not pretreated with melatonin. The data also indicated that 2.0 mM concentration of melatonin (500X less concentration) was as effective in decreasing the radiation-induced chromosome damage, as 1.0 M DMSO. This study is the first to demonstrate the ability of melatonin to significantly decrease gamma radiation-induced chromosome damage in human blood lymphocytes <u>in vitro</u>. (See **Appendix**, Manuscript #5: Vijayalaxmi et al., 1995a)

Protection Against Micronuclei Induction

Three separate experiments were conducted, each using a blood sample from three of the earlier six donors. The same experimental protocol as described in the previous investigation I was followed: from each blood sample, separate cultures were set up, treated with melatonin, exposed to gamma radiation, washed and incubated at 37+1°C in a humidified atmosphere of 5% CO_2 and 95% air. After 44 hours of incubation, cytochalasin B was added to all cultures (4 μg/ml) to block the dividing cells in cytokinesis. At the end of 72 hours, lymphocytes

were collected, treated with 0.8% sodium citrate and fixed in 5:1 methanol acetic acid mixture. Fixed cells were dropped gently onto clean microscope slides, air dried and stained with Giemsa (4%) using standard procedures. All slides were coded by an individual other than the scorer, and evaluated at 1000X magnification for the frequency of micronuclei in cytokinesis blocked binucleate cells with well preserved cytoplasm [Fenech, 1993].

After different treatments, the overall response of the lymphocytes from the three donors was similar, although the absolute values were slightly different. The expected frequencies of binucleate cells containing micronuclei and the total number of micronuclei in combined treatment groups, i.e., melatonin+150 cGy, DMSO+150 cGy and solvent control (ethanol)+150 cGy, were computed as the sum of the effects of the two individual treatments minus the frequency in the control (unirradiated and untreated). The statistical significance of the decrease in the observed frequencies relative to the expected ones was evaluated using a one-tailed test [Vijayalaxmi and Burkart, 1989]. Lymphocytes which were pretreated with melatonin and then exposed to gamma radiation exhibited a significant and melatonin concentration-dependent decrease in the frequencies of binucleate cells containing micronuclei and the total number of micronuclei, as compared with irradiated lymphocytes which were not pretreated with melatonin. Again, the data also indicated that the 2.0 mM concentration of melatonin (500X less concentration) was as effective in decreasing the radiation-induced micronuclei as 1.0 M DMSO. (See **Appendix**, Manuscript #6.

CONCLUSIONS

Induction of NF-κB

We hypothesized that the effect of ionizing radiation on the induction of the DNA binding activity of NF-κB may be attributed to different mechanisms. The signaling cascade leading to the activation of NF-κB by low-dose ionizing radiation may be initiated at the plasma membrane, as has recently been reported in UV-irradiated enucleated HeLa S3 cells [Devary, et al., 1993). Oxidants such

as H_2O_2 and superoxide, produced by ionizing radiation through radiolysis of bound and solvent water in the cells, can induce the activation PLC-γ, leading to mobilization of Ca^{++} and activation of PKC [Datta et al., 1992; Nicotera et al., 1986]. The transient activation of PKC may cause the release of I-κB from the [p50-p65-I-κB] complex, followed by increased nuclear localization of NF-κB. Alternatively, the direct action of ROIs produced by ionizing radiation can dissociate inhibitory subunit I-κB from NF-κB. The intracellular ROIs in Jurket T cells generated by the addition of micromolar concentrations of H_2O_2 were shown to activate NF-κB by inducing the release of the I-κB [Schreck et al., 1991]. On the other hand the activation of NF-κB could be mediated by an intranuclear signaling cascade. The ionizing radiation which causes DNA damage can eventually result in a reverse signaling mechanism from the nucleus to cytoplasm as a cellular response [Brach et al., 1991]. This response possibly involves the activation of PKC, resulting in translocation of NF-κB into the nucleus. A number of DNA damaging agents, such as 1-β-D-arabinofuranosylcytosine (ara-C), UV light, alkylating agents and etoposide, have been reported to induce the activation of PKC [Devary et al., 1991; Kharbanda et al., 1990; Rubin et al., 1991; Kharbanda et al., 1991]. Taken together, these observations indicate that the DNA binding activity of NF-κB induced by low-dose ionizing radiation may involve more than one signaling pathway. (See **Appendix**, Manuscript #3: Radiation Research 140:97-104.

Investigation of Melatonin as a Radioprotector

The extent of reduction in radiation-induced chromosome damage in the lymphocytes pretreated with melatonin [Vijayalaxmi et al., 1995a] was very similar to the extent of decrease observed in the incidence of micronuclei [Vijayalaxmi et al., 1995b]. These results indicate the ability of melatonin to offer protection against the genetic damage induced by gamma radiation. The data may have implications for protection of humans against genetic damage induced endogenously or exogenously by free radical producing physical and chemical mutagens and carcinogens.

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We wish to express our appreciation to the Air Force Office of Scientific Research for its support of this project. We also wish to thank the laboratory staff in the Division of Radiation Oncology who provided direct or ancillary support for this project, including Robert Klipper, Kevin Sadeghi, and Belinda Leal. We also wish to express our appreciation to Dr. Russel Reiter and his staff, of the Department of Cellular and Structural Biology, for their collaboration in the melatonin research, and to Ms. Joanne Murray, of the Department of Radiology and Ms. Pat Rosas, Division of Radiation Oncology, for report and manuscript preparation and secretarial assistance.

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APPENDIX

1. **Manuscript 1:** Prasad, A.V., Mohan, N., Sadeghi, K., Meltz, M.L. and Chandrasekar, B. (1994) Effect of high cell density stress on the expression of transcription factor NF-κB. Toxic Substance Journal 13, 159-170.

2. **Manuscript 2:** Prasad, A.V., Mohan, N., Chandrasekar, B. and Meltz, M.L. (1994) Activation of Nuclear Factor κB in Human Lymphoblastoid Cells by Low-Dose Ionizing Radiation. Radiation Research 138, 367-372.

3. **Manuscript 3:** Mohan, N. and Meltz, Ml.L. (1994) Induction of Nuclear Factor κB After Low-Dose Ionizing Radiation Involves Reactive Oxygen Intermediate Signaling Pathway. Radiation Research 140, 97-104.

4. **Manuscript 4:** Prasad, A.V., Mohan, N., Chandrasekar, B. and Meltz, M.L. (1994) Induction of "Immediate Early Genes" by Low Dose Ionizing Radiation. Radiation Research, Revised and Resubmitted.

5. **Manuscript 5:** Vijayalaxmi, Reiter, R.J. and Meltz, M.L. (1995) Melatonin protects human blood lymphocytes from radiation-induced chromosome damage. Mutation Research 346, 23-31.

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EFFECT OF HIGH CELL DENSITY STRESS ON THE EXPRESSION OF TRANSCRIPTION FACTOR NF-κB

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Nuclear factor NF-κB is involved in the transcriptional regulation of several genes. Our data in 244B human lymphoblastoid cells demonstrate for the first time that NF-κB expression was affected by cell density in cells treated with phorbol 12-myristate 13-acetate (PMA). When the cell density was 1 × 10^6 cells/ml, the expression was maximum at 8 h: the expression at this time was less at the higher cell densities of 2.5 and 5.0 × 10^6 cells/ml. The expression was dependent on the time at which the cells were harvested, with higher levels being observed a second time at 36 h, following negligible expression for all 3 cell densities at 16 h. NF-κB subunit p50 expression was highest at the lowest cell density, while p65 expression was unaltered at the three cell densities.

INTRODUCTION

Cell density effects on the regulation of specific gene expression in different cells have been demonstrated (Kumatori et al., 1991; Kiel et al., 1992; Merenmies, 1992; Phillips et al., 1992; Rollins et al., 1987). For example, the expression of heparin-binding growth-associated mol-

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ecule (HB-GAM), which acts on cell growth and differentiation (Merenmies & Racivala, 1990), was reported to be enhanced with increasing cell density (Merenmies, 1992). As another example, the proto-oncogenes *c-fos* and *c-myc* were reported to be differentially expressed (Kumatori et al., 1991; Kiel et al., 1992; Merenmies, 1992; Phillips et al., 1992; Rollins et al., 1987) at high cell density. Increased extracellular thiol production at high cell density was also reported in transformed RAW 264.7 mouse macrophages treated with lipopolysaccharide (LPS), and the existence of a redox stress transponding system was discussed (Toledano & Leonard, 1991). The mechanism(s) underlying these phenomena have not yet been determined.

The DNA-binding protein NF-κB is a pleiotropic transcription factor that induces the transcription of specific late-response genes (Baeuerle & Baltimore, 1991; Leonardo & Baltimore, 1989). It exists as a heterodimer (a p50 and p65 complex) in the cytoplasm in an inactive form, complexed to an inhibitor IκB. On dissociation from IκB by various mechanisms, it translocates to the nucleus and stimulates targeted cellular genes (Baeuerle & Baltimore, 1988a, 1988b; Staal et al., 1990; Ghosh & Baltimore, 1990). The study of gene regulation pathways is of obvious importance, and experimental protocols should be designed to optimize the opportunity to observe the biological events.

The objective of the present study was to determine whether high cell density would interfere with the induction of NF-κB transcription factor. We have observed, and are reporting for the first time, that NF-κB transcription factor expression can be effected by cell density. Our data demonstrate that maximal NF-κB expression is decreased at high cell density in Epstein-Barr virus (EBV) transformed human lymphoblastoid B cells (the 244B cell line) treated with phorbol 12-myristate 13-acetate (PMA) and, more obviously, that the time course of NF-κB induction and its subunit regulation are also affected.

MATERIALS AND METHODS

Cell Culture and Preparation of Nuclear Extracts

Epstein-Barr virus-transformed 244B human lymphoblastoid cells, originally supplied to our laboratory by Dr. J. Schwartz (University of Chicago), were cultured in RPMI-1640 medium containing 2 m*M* L-glutamine and 10 m*M* HEPES (Mediatech, Inc., Herndon, Va.) and supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and 50 μg/ml gentamicin (Sigma, St. Louis, Mo.). The population doubling time of the cells under our standard growth conditions is 33–36 h. The cells used in these experiments were always collected at 72 h after initiation of the cell culture, when they had achieved a density of ap-

proximately 1×10^6 cells/ml. Cell viability was determined using the trypan blue dye exclusion method at the start of each experiment; it was always ≥98%. Prior to the experiments the cells were washed, counted (Coulter Electronics, Inc., Hialeah, Fla.), and transferred to fresh complete media and preincubated for 30 min at 37°C in humidified incubators with 5% CO_2/95% air in T-25 flasks (Corning, Corning, N.Y.). The cells were distributed at cell densities of 1, 2.5, and 5×10^6 cells/ml in a 6-ml volume. Incubations for various time periods (1–72 h) were then initiated with the addition of 20 ng/ml PMA (Sigma) to each flask; appropriate controls without PMA were simultaneously incubated. After incubation, the cells were harvested from culture medium by centrifugation for 5 min at 250 × g and resuspended in phosphate-buffered saline (PBS); they were then washed twice with PBS in a microcentrifuge (Eppendorf, model 5415C, rotor S-45-18-11, Westbury, N.Y.) at 14,000 rpm. The resulting pellet was stored at −70°C. Nuclear extracts were prepared using a modification (Hillman et al., 1992) of the Dignam et al. (1983) protocol. Protein concentrations were determined using the bicinchonic acid (BCA) method and measured with an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR5000, Chantilly, Va.).

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were performed using an NF-κB binding-protein detection system (Promega, Madison, Wis.). The manufacturer's protocol was followed with slight modification. A double-stranded oligonucleotide (5 ng) containing a tandem repeat of the consensus sequence for the NF-κB DNA binding site, -GGGGACTTTCC-, was end-labeled with 100 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham, Arlington Heights, Ill.). An aliquot of the labeled probe (30,000 cpm) was added to the nuclear extract (15 μg) and 3 μg of poly(dI-dC) (Pharmacia Fine Chemicals, Nutley, N.J.) in binding buffer; the mixture was incubated for 20 min at 22°C. For confirmation of the specific binding of NF-κB, the preceding reaction was simultaneously performed with the addition of labeled probe (30,000 cpm) to nuclear extract that had been preincubated with homologous unlabeled NF-κB-specific oligonucleotide probe for 5 min on ice. All samples were subsequently electrophoresed (150 V) (Hoefer Scientific, San Francisco, Calif.) through 6% polyacrylamide gel (acrylamide : bis w/w, 29 : 1) in Tris-glycine buffer (25 m*M* Tris, 0.19 *M* glycine, and 1 m*M* EDTA, pH 8.0) until the time required for the free probe to migrate to the bottom of the gels. The gels were dried and autoradiographed at −70°C with intensifying screens on hyperfilm (Amersham, Arlington Heights, Ill.).

Antibodies/Western Blotting

The specific affinity-purified rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) used in this study were (1) rabbit anti-human NF-κB p50 and (2) rabbit anti-human NF-κB p65. These antibodies do not cross-react with each other. Immunoblotting was carried out essentially as described by Korc et al. (1992). In brief, 30-μg protein samples (an aliquot of nuclear extract prepared for EMSA) were subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were then electroblotted onto nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.) at 30 mA overnight. Nonspecific protein binding was blocked with 10% normal goat serum (Kirkegaard & Perry Labs, Inc., Gaithersburg, Md.). The membranes were then incubated for 18 h at 4°C under continuous agitation with the respective primary antibody (5 μg/ml). After incubation, the membranes were washed with a buffer containing 20 m*M* Tris (pH 7.5), 500 m*M* NaCl, and 0.05% v/v Tween-20, and then again incubated for 2 h with goat anti-rabbit immunoglobulin G (IgG) (1 : 2500 dilution), followed by incubation for 2 h with [^{125}I]protein A (0.33 μCi/ml; Amersham). The blots were washed, dried, and analyzed by autoradiography.

Quantitative Analysis

The autoradiograms were analyzed using a Betascope 603 blot analyzer (Betagen Division of Intelligenetics, Inc., Mountain View, Calif.). The slow-moving band was separately quantitated from the total, which was also quantitated. The area analyzed for each band was kept constant for all of the bands in an autoradiogram.

RESULTS AND DISCUSSION

Effect of Cell Density on NF-κB Expression

The initial experiment (using EMSA) examined the constitutive level of NF-κB over time at the lowest cell density (1×10^6 cells/ml), and PMA-induced levels over time at three cell densities. The cells were collected for analysis at 1, 8, 16, 36, and 72 h in culture (Fig. 1). Visual examination indicated the possibility of variation in the constitutive level at the different time points. At the 1×10^6 cells/ml density, a maximum was observed at 8 h. When the PMA-induced levels were compared to the level observed at 1 h of induction for that cell density, a maximum appeared to be present for the 2.5×10^6 cell density at 72 h. For the 5×10^6 cells/ml, compared to the 1-h time point at this density, the maximum possibly occurred at 16 or 36 h.

Since the initial experiment was not quantitative, the experiment was independently repeated with constitutive levels determined simultaneously for each cell density at each time point of 1, 8, 16, and 36 h after initiation of PMA treatment. The EMSA technique initially used was altered to allow for increased separation of the slower moving band (area of specific binding of NF-κB) from the faster moving band (with nonspecific binding). Quantitation was performed by utilizing Betascope blot analysis. The autoradiograms are shown in Figure 2A, with analyses for the slow-moving (specific NF-κB binding) region presented in Table 1. The specificity is demonstrated in the Figure 2 insert (C).

In the non-PMA-treated constitutive controls, at all times and densities, variations were present but were relatively small, with the only evidence for a considerable change (2.2-fold) at 8 h for the 1×10^6 cells/ml density. This is likely to be experimental variation, and is not supported by the first experiment (Fig. 1).

The results of PMA induction of NF-κB in the second experiment are summarized in Table 1. At all three cell densities, a small amount of expression appears at the 1-h time point. The highest induced level of NF-κB (cpm above constitutive level) was seen at the 8-h incubation time at all three densities. This is consistent with the observation of Granelli-Piperno and Nolan (1991) for NF-κB activation at 6 h in primary human T cells, and also with our own observation in the first experiment (Fig. 1). At 1×10^6 cells/ml, the expression declined to the constitutive level at 18 h, but then showed another high value at 36 h after addition of the PMA. Similar biphasic patterns of NF-κB expression were observed at cell densities of 2.5 and 5×10^6 cells/ml (Fig. 3), where the induced level (cpm) at 36 h was only one-half of the 8-h value. In Table 1, we have also calculated the ratio (column d) of the induced expression (cpm above the constitutive level) to the value of the constitutive level, and also the fold stimulation (column e), the ratio of the PMA-treated expression to the constitutive control level. While the use of either of the latter two ratios also shows the biphasic nature of the induction over time for all three cell densities, the maximum induction at the lowest cell density is not evident. This is clearly a function of the constitutive value at this particular time point.

The effect of cell density at the different times after PMA addition was examined. At 1 h, the induced level appears to show a slight increase with increasing cell density (column c of Table 1). At 8 h, when the induction is maximum (for each cell density), the greatest induction above constitutive level is at the lowest cell density; it is 20% less at 2.5×10^6 cells/ml and 6% less at 5.0×10^6 cells/ml (Table 1, column c). At 18 h, there is very little activation at any of the cell densities examined. At 36 h, where there is a second high level, it is obvious at both 2.5×10^6 and 5×10^6 cells/ml that a significant reduction in the extent

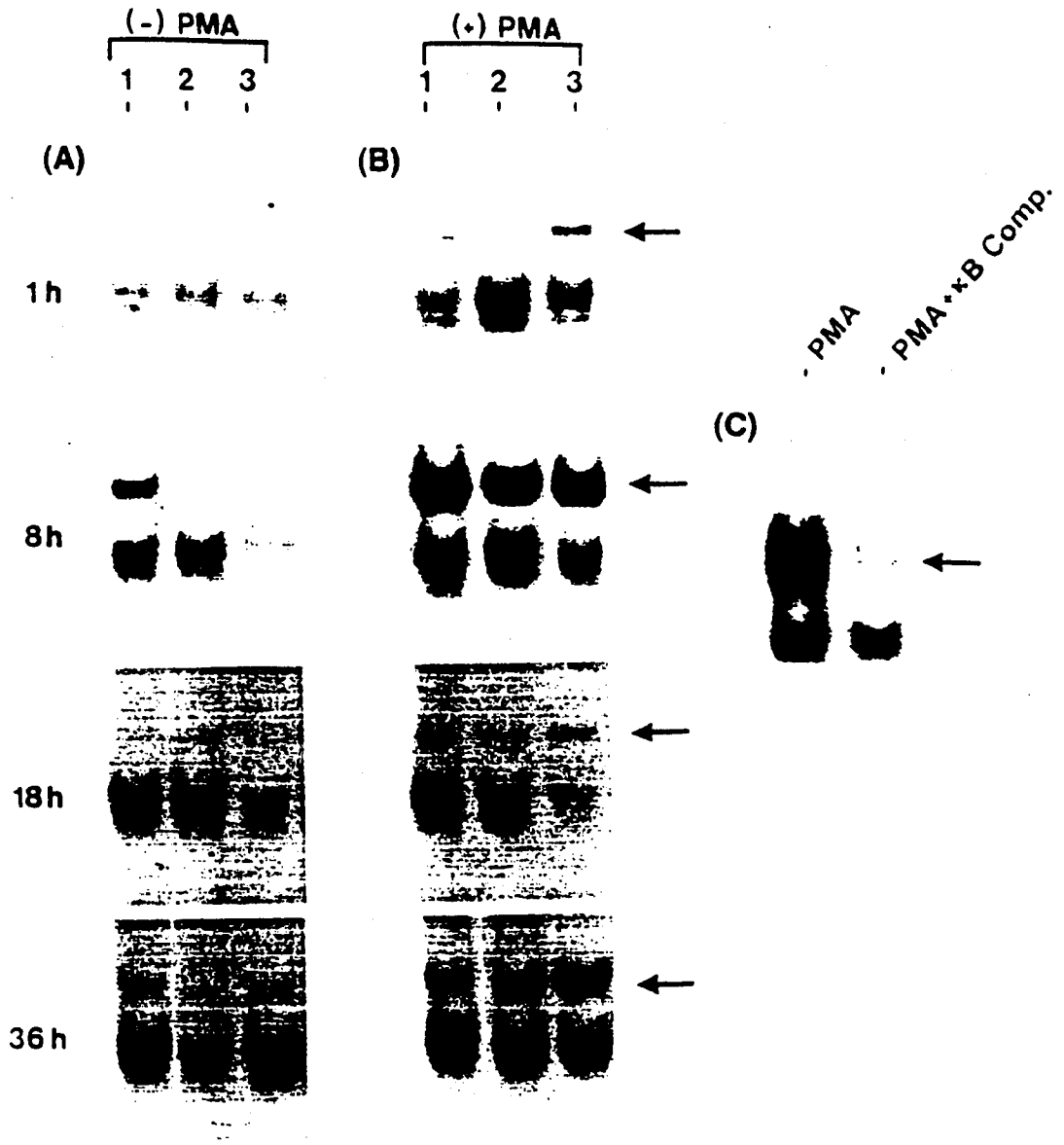

Figure 2. Density-dependence and time kinetics of NF-κB protein expression in 244B human lymphoblastoid cells. An electrophoretic mobility shift analysis was performed using equal amount of protein (15 μg) from nuclear extract of 244B cells derived from 1.0, 2.5, and 5.0 × 10^6 cells/ml. (A) Cells were cultured in the absence of PMA and harvested at 1, 8, 18, and 36 h. (B) Cells were cultured in the presence of PMA (20 ng/ml) and harvested at 1, 8, 18, and 36 h. Arrow represents the position of gel shift complexes. (C) A competition assay to test the specific binding of NF-κB was performed by incubating the nuclear extracts (15 μg protein) in the presence or absence of 250 molar excess of homologous unlabeled NF-κB specific ds-oligonucleotide competitor for 5 min on ice, followed by the addition of γ-^{32}P-labeled NF-κB specific probe. A representative complex tested for specific binding of NF-κB is shown by the arrow.

Table 1. Cell density and time-dependent activation of NF-κB

Cell density (1×10^6 cells/ml)	Time (h)	(a) (−)PMA (cpm)	(b) (+)PMA (cpm)	(c) Induced level* (cpm) (b)-(a)	(d) Ratio of induced to constitutive level (c)/(a)	(e) Fold stimulation (b)/(a)
1	1	121	185	64	0.53	1.5
	8	266	774	508	1.91	2.9
	18	86	109	23	0.27	1.3
	36	103	525	422	4.09	5.1
2.5	1	123	205	82	0.67	1.7
	8	155	560	405	2.61	3.6
	18	118	169	51	0.43	1.4
	36	154	357	203	1.32	2.3
5.0	1	113	245	132	1.17	2.2
	8	104	581	477	4.59	5.6
	18	79	134	55	0.70	1.7
	36	119	347	228	1.92	2.9

*Induced with 20 ng/ml PMA.

of expression above the constitutive level has occurred (52 and 46%, respectively; Table 1, column c). For the higher cell densities, the use of the ratios of induced to constitutive levels (column d) or the fold stimulation (column e) supports this observation. If, however, these ratios had been used for comparison at the 1×10^6 cells/ml density, the patterns of response would have been considerably different. Based on the methodologies we have used, we do not believe that this is the case; rather, the greatest expression at 8 h occurs at the lowest cell density.

Expression of NF-κB Subunit p50 and p65 at Different Cell Densities

The NF-κB subunit p50 and p65 regulation were investigated (using their respective antibodies and western blot analysis) at the maximum time point of 8 h in the presence or absence of PMA. Non-PMA-treated cells served as a negative control. The results (Fig. 4, upper panel) demonstrate the abundance of the p50 subunit of NF-κB at the cell density of 1×10^6 cells/ml (both constitutive and PMA-induced), with lower levels at the densities of 2.5 and 5×10^6 cells/ml. In contrast, the p65 subunit was unaffected by PMA treatment at all three cell densities (Fig. 4, lower panel).

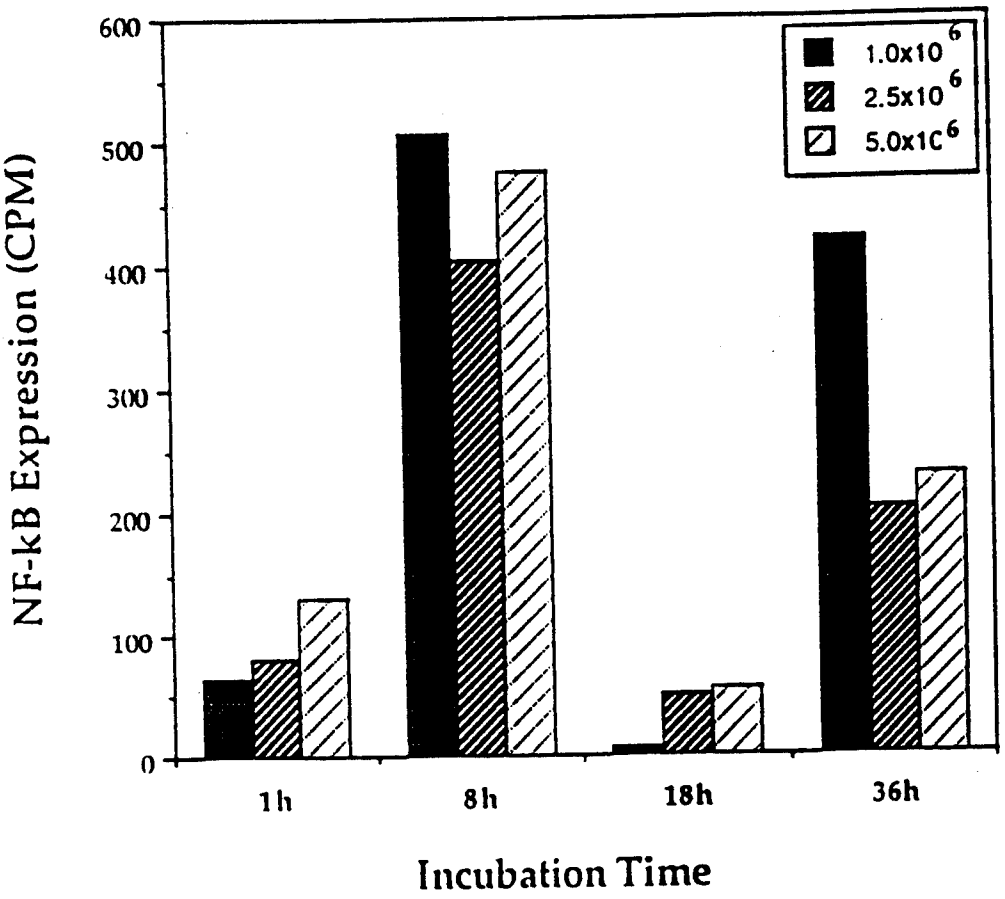

Figure 3. Betascope blot analysis of the dried gel of the autoradiogram shown in Figure 2B. Quantitative analysis of NF-κB activity from PMA-treated (20 ng/ml) cells is expressed as relative cpm levels.

While our data showing the presence of both subunits in the nucleus are in good agreement with other published observations (Angel et al., 1988; Baeuerle & Baltimore, 1988a, 1989) demonstrating the need for both p50 and p65 subunits in complex to activate the NF-κB DNA binding activity, we have observed further that the nuclear localization of NF-κB subunit p50 into the nucleus (at 8 h) is modulated by the cell density, decreasing with increased cell density, whereas the p65 subunit available in the nucleus was unaltered with increasing cell density. It is widely accepted that the NF-κB DNA binding activity requires the disassociation of inactive I-κB in the cytoplasm prior to the translocation of the p65–p50 heterodimer into the nucleus (Baeuerle & Baltimore, 1988a, 1988b, 1989). The presence of the heterodimer in the nucleus was greatest at the lowest cell density and lowest at the highest cell density. This could be due to the high cell density stress release "a factor" (Kiel et al., 1992), denoted ADF (Wakasugi et al., 1990), which could inhibit the disassociation of p50–p65 complex from I-κB. At the

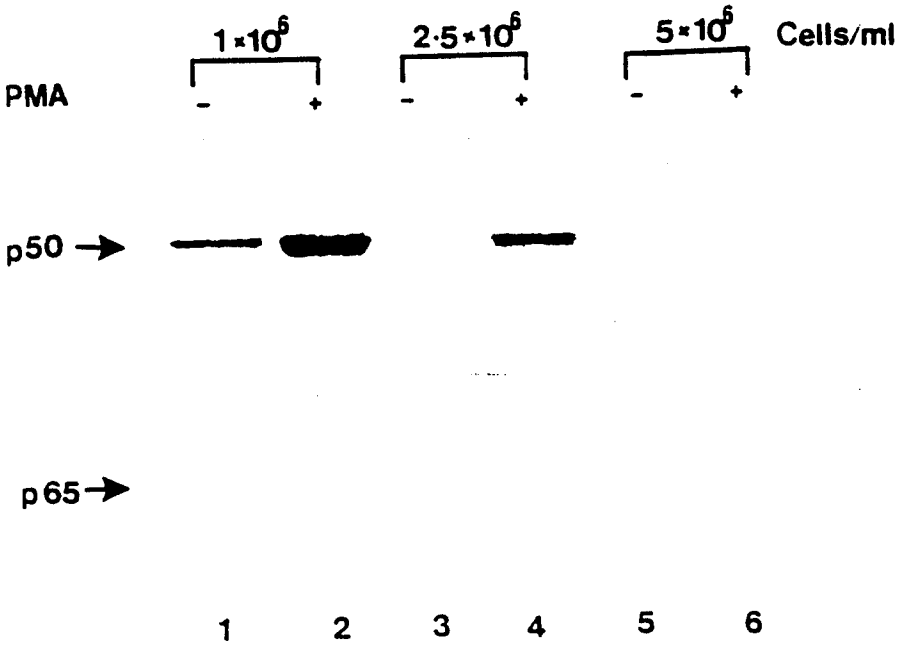

Figure 4. Western blot analysis of 244B cells cultured in the presence or absence of PMA (20 ng/ml) at the indicated cell densities and harvested at 8 h. Protein samples (30 μg) from the nuclear extract prepared for EMSA were analyzed by SDS-PAGE under reducing condition, and electroblotted onto nitrocellulose membrane. The immobilized proteins were then processed according to the protocol described under Materials and Methods. After washing, the membrane was autoradiographed. The specificity of antibodies is characterized by incubating with their respective peptides.

same density at which the heterodimer availability in the nucleus was lowest, the p50 subunit availability was also lowest.

In conclusion, our results show evidence for the first time that the density of the cells during in vitro culture can influence the time of appearance of transcription factor NF-κB in the nucleus upon PMA induction. A second novel phenomenon observed was that at any cell density, the level of induced NF-κB in the nucleus was dependent on the incubation time, with a second high level occurring at 36–72 h, depending on the cell density. We have also observed that the nuclear localization of NF-κB subunit p50 was modulated by the cell density, decreasing with increased cell density, whereas the p65 subunit was unaltered with increasing cell density.

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RADIATION RESEARCH 138, 367–372 (1994)

Activation of Nuclear Factor κB in Human Lymphoblastoid Cells by Low-Dose Ionizing Radiation

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Prasad, A. V., Mohan, N., Chandrasekar, B. and Meltz, M. L. Activation of Nuclear Factor κB in Human Lymphoblastoid Cells by Low-Dose Ionizing Radiation. *Radiat. Res.* **138**, 367–372 (1994).

Nuclear factor κB (NF-κB) is a pleiotropic transcription factor which is involved in the transcriptional regulation of several specific genes. Recent reports demonstrated that ionizing radiation in the dose range of 2–50 Gy results in expression of NF-κB in human KG-1 myeloid leukemia cells and human B-lymphocyte precursor cells; the precise mechanism involved and the significance are not yet known. The present report demonstrates that even lower doses of ionizing radiation, 0.25–2.0 Gy, are capable of inducing expression of NF-κB in EBV-transformed 244B human lymphoblastoid cells. These results are in a dose range where the viability of the cells remains very high. After exposure to ^{137}Cs γ rays at a dose rate of 1.17 Gy/min, a maximum in expression of NF-κB was seen at 8 h after a 0.5-Gy exposure. Time-course studies revealed a biphasic time-dependent expression after 0.5-, 1- and 2-Gy exposures. However, for each time examined, the expression of NF-κB was maximum after the 0.5-Gy exposure. The expression of the p50 and p65 NF-κB subunits was also shown to be regulated differentially after exposures to 1.0 and 2.0 Gy.

INTRODUCTION

Recent studies have demonstrated that ionizing radiation induces the expression of several specific genes, including tumor necrosis factor (TNF)-α, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), interleukin 1 (IL-1) and also the c-*fos/jun* proto-oncogenes (*1–7*). The induction has been attributed either to the activation of the protein kinase C (PKC) signaling pathway or through the reactive oxygen intermediates pathway (*1, 2*). Although the precise mechanism responsible for the specific gene expression is still unclear, at least one laboratory has attempted to demonstrate the involvement of transcriptional regulation (*4, 8*). Transcriptional modulation, which plays a major role in the repair of DNA damage, proliferation and other cellular functions, requires the activation of transcription factors that bind to specific DNA sequences (*9*).

Nuclear factor κB (NF-κB) is a pleiotropic transcription factor available in the cytoplasm as a p50 and p65 heterodimer complexed to a regulatory inactive subunit I-κB (*10*). The disassociation of the heterodimer from I-κB precedes its translocation into the nucleus (*11, 12*). This event can be mediated by PKC activation (*13, 14*) or a reactive oxygen intermediates signaling cascade (*15, 16*). NF-κB recognizes and binds an 11-bp specific DNA sequence in the enhancer region of the κ immunoglobulin light chain (*17*). Previous reports of expression of NF-κB after exposure of C3 and C5 ABR lymphoblastoid cells (*18*) and human myeloid leukemia cells (*19*) to ionizing radiation involved high doses, ranging from 2–50 Gy, after which significant cell death will occur (*20*). In this report we have demonstrated that expression of NF-κB occurs at the lower-dose exposures of 0.25, 0.5, 1 and 2 Gy, after which cell viability is high. We also describe the time course of the NF-κB expression and its p50 and p65 subunit expression. These results were obtained in cells potentially representative of the human immune system, the Epstein-Barr virus (EBV)-transformed 244B human lymphoblastoid cell line.

MATERIALS AND METHODS

Cell Culture

EBV-transformed 244B human lymphoblastoid cells (originally supplied by Dr. J. Schwartz) were cultured in complete RPMI-1640 medium containing 2 m*M* L-glutamine and 10 m*M* HEPES (Mediatech, Inc., Herndon, VA) and supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 50 μg/ml gentamicin. The cells used in these experiments were always collected 72 h after initiation of the cell culture, when they had achieved a density of approximately 1×10^6 cells per ml. For each experiment cell viability was determined by the trypan blue dye exclusion method; it was always ≥98%. The cells were transferred to fresh complete medium at a density of 1×10^6 cells/ml and preincubated for 30 min at 37°C in a humidified incubator with 5% CO_2/95% air in T-25 flasks

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(Corning, Corning, NY) prior to exposure to ionizing radiation or phorbol 12-myristate 13-acetate (20 ng/ml) (PMA; Sigma, St. Louis).

Exposure and Treatment

Exponentially growing cells were removed from the 37°C incubator and immediately exposed at room temperature to ^{137}Cs γ rays at a dose rate of 1.17 Gy/min; the doses ranged from 0.25–2.0 Gy. The irradiations were performed in an Atomic Energy of Canada Ltd. Gamma Cell-40 irradiator. For time-course studies, the cultures were incubated immediately after exposure at 37°C and harvested at 1, 8, 16, 24, 36 and 72 h.

Cell Harvest

After incubation, the cells were harvested from culture medium by centrifugation for 5 min at 250*g* and resuspended in phosphate-buffered saline (PBS); they were then washed twice with PBS in a microcentrifuge (Eppendorf, Model 5415C, Rotor S-45-18-11, Westbury, NY) at 14,000 rpm. The resulting pellet was stored at −70°C.

Electrophoretic Mobility-Shift Assays

Nuclear extracts were prepared from the harvested cells using a modification (*21*) of the protocol of Dignan *et al.* (*22*). Protein concentrations were determined using the bicinchonic acid (BCA) method following the manufacturer's protocol (Pierce, Rockford, IL). Fluorescence was measured with an ELISA plate reader (Dynatech MR5000, Chantilly, VA).

The electrophoretic mobility-shift assay was performed using an NF-κB binding protein detection system following the manufacturer's protocol (Promega, Madison, WI) with slight modification. Briefly, a double-stranded oligonucleotide containing a tandem repeat of the consensus sequences of -GGGGACTTTCC- was end-labeled with T_4 polynucleotide kinase. The binding reaction was performed by mixing 20 μg of nuclear extract, 3 μg of poly(dI-dC) (Pharmacia Fine Chemicals, Nutley, NJ), γ-^{32}P(ATP)-labeled oligonucleotide probe (30,000 cpm) and T_4 polynucleotide kinase in binding buffer and then incubating for 20 min at 22°C. For the competition assay the nuclear extract (20 μg) was preincubated with homologous unlabeled NF-κB oligonucleotide for 5 min on ice, followed by the addition of ^{32}P end-labeled NF-κB probe. All samples were subsequently electrophoresed through 6% polyacrylamide gel (acrylamide:bis w/w 29:1) in Tris-glycine buffer (25 m*M* Tris, 0.19 *M* glycine and 1 m*M* EDTA). Electrophoresis was performed (at 150 V) until the time required for the free probe to migrate to the bottom of the gels (Hoefer Scientific, San Francisco, CA). The gels were dried and autoradiographed at −70°C with intensifying screens on hyperfilm (Amersham, Arlington Heights, IL).

Antibodies/Western Blotting

Protein samples (30 μg) separated by 7.5% SDS-PAGE were electroblotted onto nitrocellulose membrane (Grade BA 85, Schleicher and Schuell, Keene, NH) at 30 mA overnight as described by Korc *et al.* (*23*). The membrane was then incubated with rabbit polyclonal anti-human NF-κB p50 or NF-κB p65 primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (5 μg/ml) for 18 h at 4°C under continuous agitation. Nonspecific protein binding was blocked with 10% normal goat serum (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The membrane was then washed at room temperature in a buffer containing 20 m*M* Tris (pH 7.5), 500 m*M* NaCl and 0.05% v/v Tween-20 and incubated at room temperature for 2 h with goat anti-rabbit IgG (1:2500 dilution). This was followed by a 2-h incubation (room temperature) with ^{125}I-protein A (12.21 kBq/ml; Amersham). The blots were washed, dried and analyzed by autoradiography.

Densitometric Analysis

Autoradiograms were analyzed by using a desktop digital imaging method (*24*). Briefly, the autoradiograms were photo-screened by standard video imaging equipment connected to a Macintosh computer (Model IIci) and the image was analyzed using an NIH 1.45 image analysis software package with an integrated density program. The area analyzed for each band was kept constant for all the bands in an autoradiogram. Background density on an autoradiogram was subtracted from the densitometric data of each band.

RESULTS

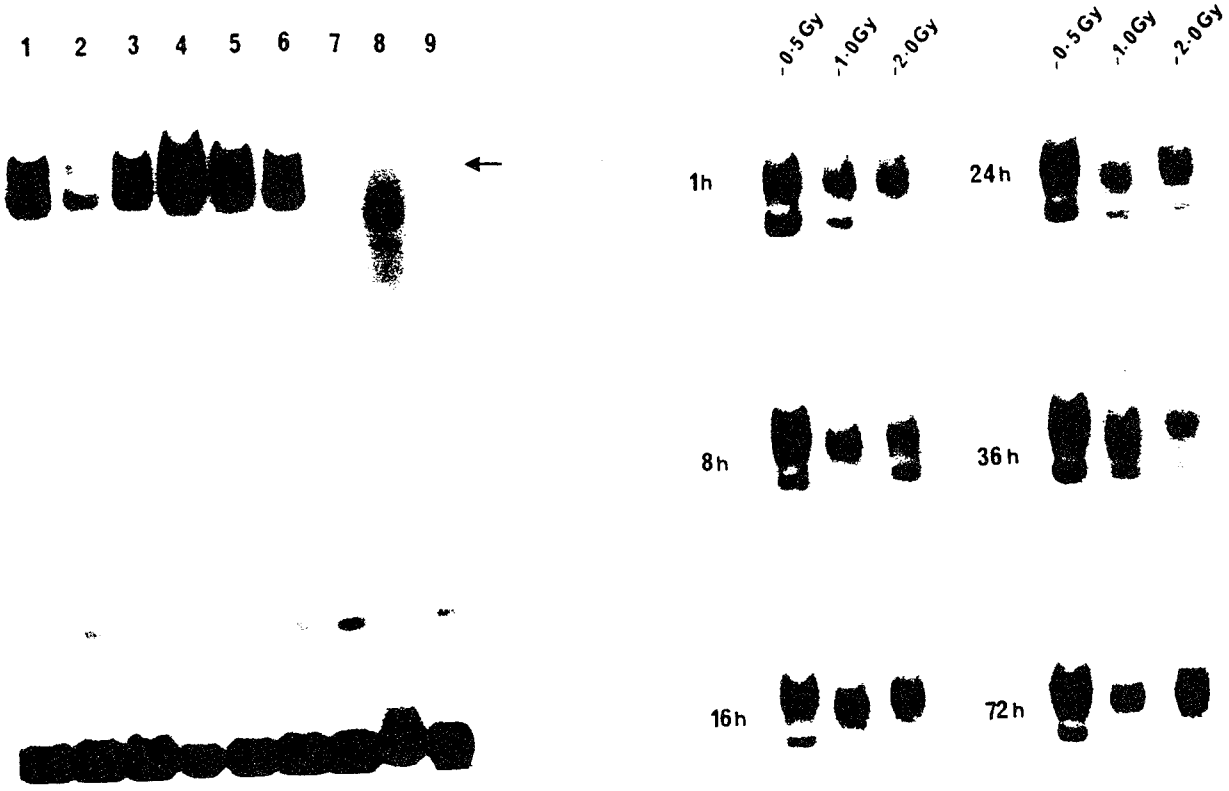
Dose–Response Expression of NF-κB

In an initial study, when the expression of NF-κB was observed at 24 h after exposure, it was detected at a dose of 0.25 Gy (Fig. 1, lane 3). The magnitude of this induction reached a maximum at 0.5 Gy (lane 4) and then decreased at the doses of 1.0 and 2.0 Gy (Fig. 1A). Both the PMA-treated and untreated controls acted as expected; i.e., PMA-treated samples exhibited a 2-fold increase in expression of NF-κB when compared to the constitutive levels in untreated controls. Densitometric analysis revealed (Fig. 1B) that the expression after 24 h at 0.25, 0.5, 1.0 and 2.0 Gy was 1.8-, 2.8-, 2.2- and 1.6-fold greater than that of the basal level expression, respectively.

Time-Course of Expression of NF-κB

Since the expression of NF-κB was found to be dependent upon dose at 24 h after incubation, we investigated the time course of its expression after the doses of 0.5, 1 and 2 Gy. The intervals selected included 1, 8, 16, 24, 36 and 72 h after exposure. The data are presented in Fig. 2. When the expression at each time after a dose was compared to the 1-h value for that dose, expression of NF-κB after 0.5 Gy reached its maximum (1.6-fold) at 8 h, decreased (1.2-fold) by 16 h, started increasing (1.3-fold) again at 24 h and reached a second maximum (1.5-fold) by 36 h; it was at this same level at 72 h. After both the 1- and 2-Gy exposures, a maximum did not occur at 8 h; the expression increased after both doses to a maximum (1.2-fold) at 16 h. After the 1-Gy exposure, it was decreased at 24 h and again increased (1.2-fold) at 36 h, with a decrease at 72 h. After the 2-Gy exposure, the expression was decreased at 24 h, but continued decreasing to a minimum at 36 h; it again rose to its maximum (1.2-fold) at 72 h. The level of expression is clearly dependent on time after all three doses.

When the fold comparison was made from the densitometric analysis between the doses, at each time after exposure, the greatest expression was observed after a dose of 0.5 Gy. At 8 and 36 h, the expression was higher after 1 Gy than after 2 Gy, i.e., 1.2- and 1.4-fold greater, respectively. In contrast, at 24 and 72 h, the expression after 2 Gy was 1.2-fold greater than that of 1 Gy. In other experiments conducted in our laboratory comparing PMA-induced expression of NF-κB to mock-irradiated and non-PMA-treated controls, little variation in the very low constitutive levels detected was observed for incubation times of 1, 8, 18 and 36 h (*25*). The constitutive level would therefore have little impact on the relative changes described here, in light of the low constitutive level again seen in Fig. 1.



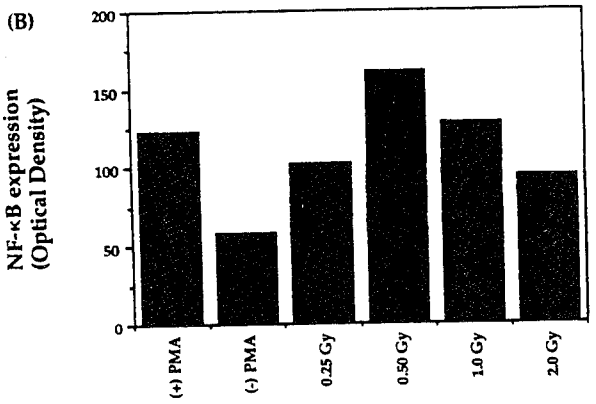


FIG. 1. Panel A: Dose-dependent activation of NF-κB by low-dose ionizing radiation. Electrophoretic mobility-shift assay was performed by incubating the nuclear protein (20 μg) obtained from 24 h culture of 244B cells with γ-^{32}P(ATP)-labeled (30.000 cpm) NF-κB specific ds-oligonucleotide on 6% polyacrylamide gel. Autoradiogram of native gel is shown. Lanes 1 and 2: Nuclear extract derived from the cell in the presence (lane 1) or absence (lane 2) of PMA; lanes 3–6: nuclear extract derived from the cells exposed to 0.25 Gy (lane 3), 0.5 Gy (lane 4), 1.0 Gy (lane 5) and 2.0 Gy (lane 6); lane 7: nuclear extract (20 μg) was pre-incubated with homologous unlabeled NF-κB specific ds-oligonucleotide competitor for 5 min on ice followed by the addition of ^{32}P end-labeled NF-κB probe; lane 8: HeLa cell extract as positive control; lane 9: free probe. The arrowhead indicates NF-κB specific binding region in the 244B cells. Panel B: Quantification of total NF-κB activity in the nucleus was measured by densitometric analysis of the autoradiogram shown in Fig. 1A and is represented as relative optical density values.

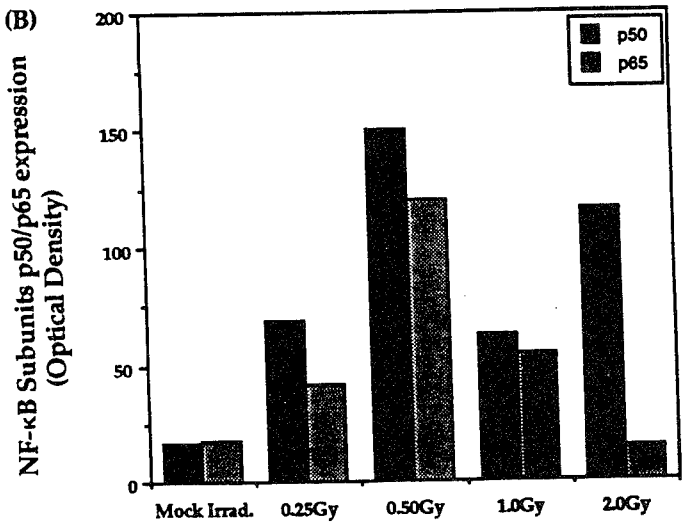


FIG. 2. Panel A: Kinetics with increasing time of binding of NF-κB from 244B cells treated with doses of ionizing radiation of 0.5, 1.0 and 2.0 Gy for the times as indicated. Twenty micrograms of nuclear protein was incubated with end-labeled double-stranded oligonucleotide containing the NF-κB binding site as described in Materials and Methods. Autoradiograms of native gel are shown. Panel B: Quantitative analysis of the autoradiogram shown in Fig. 2A and expressed as fold stimulation of NF-κB activity in comparison to value at 1 h after exposure.

being 2 Gy. In the latter study, the maximum was reached at 5 Gy, and was shown to persist at similar levels up to doses as high as 50 Gy (*19*).

Secondly, we have demonstrated that the expression of NF-κB after exposure to low-dose ionizing radiation is dependent on time. After 0.5 Gy, expression was maximum at 8 h in a first phase of response and at 36 h in a second phase of response. After exposures of 1 and 2 Gy, the maximum expression in the first phase of response occurred at 16 h and then at 36 and 72 h, respectively, in the second phase of response. This biphasic nature of expression of NF-κB over time remains unexplained; interestingly, a biphasic induction has been reported for TGF-β1 in pig skin after exposure to ionizing radiation (14–140 Gy) (*31*). Those observations were made over a much longer time, and potentially address a tissue response which may or may not be secondary to the type of short-term cellular response reported here. Our study also revealed that the maximum activation of NF-κB at any given time after exposure is after the dose of 0.5 Gy (in comparison to after 1 or 2 Gy). This contrasts with another study where, after exposure to the much higher dose of 20 Gy, the maximum DNA-binding activity of NF-κB was shown to occur much earlier after exposure (*19*). In that study, it was reported as early as 15 min after exposure and reached a maximum level at 2–4 h; it then declined rapidly to constitutive levels after 4 h. These additional differences between a low-dose-range response and a high-dose-range response suggest that different mechanisms may be involved at the different exposure levels. It must be remembered that for optimal concentrations of induction of expression of NF-κB by a chemical agent, such as upon PMA treatment, the NF-κB activation was shown to be maximal at 6 to 8 h after treatment (*32*). As is the case in our study of ionizing radiation in the low-dose range, this PMA treatment leaves cells at high viability.

Thirdly, we have also shown for the first time a differential regulation of NF-κB subunit protein levels after exposure to low-dose ionizing radiation. In untreated controls both subunit levels were expressed equally at a constitutive level. While the p65 subunit expression did increase with dose at a selected time (8 h) after exposure, the increased expression of the p50 subunit was increased substantially more. In addition, both subunit expressions were maximum at 0.5 Gy compared to the doses of 0.25, 1.0 or 2.0 Gy. Our findings, that the presence of the heterodimers (p50/p65) in the nucleus was greatest after 0.5 Gy, are consistent with our results using the electrophoretic mobility-shift assay showing maximum DNA binding of the NF-κB complex after 0.5 Gy. This differential regulation of NF-κB subunits by low-dose ionizing radiation could be due to the differential activation of pre-existing protein and/or differential induction at the level of gene expression (mRNA synthesis) of the subunit precursors of NF-κB. In support of the latter hypothesis, high doses of ionizing radiation were recently shown to cause a transient increase in NF-κB mRNA levels (*19*). More studies are required to compare the observations at the lower and higher dose ranges, in terms of both response and mechanism.

In conclusion, this study has demonstrated, for the first time, that low doses of ionizing radiation modulate NF-κB DNA-binding activity. Three experiments performed independently include: (1) dose responses for three different doses at 8 h after the exposure (Fig. 1), (2) dose responses for four different doses at six different incubation times (Fig. 2), and (3) the responses for NF-κB subunits at 8 h, compared to the control constitutive level (Fig. 3). The modulation by irradiation was dose-dependent within the low-dose range and dependent on the time after exposure. This dependence is biphasic over time. Further, the low-dose ionizing radiation also differentially regulates NF-κB subunit p50 and p65 expression.

The doses of 2–20 Gy induce high levels of radical formation, DNA damage, cell growth and progression delays, mutation, neoplastic transformation and cell killing (*26*). The lower dose range in our study can also result in all of these effects, but cell killing is minimal. The relationship of our observations at low doses, and of others (*19, 27*) at high doses, to any and all of these phenomena, as well as cell function, remains to be determined.

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Induction of Nuclear Factor κB after Low-Dose Ionizing Radiation Involves a Reactive Oxygen Intermediate Signaling Pathway

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Mohan, N. and Meltz, M. L. Induction of Nuclear Factor κB after Low-Dose Ionizing Radiation Involves a Reactive Oxygen Intermediate Signaling Pathway. *Radiat. Res.* 140, 97–104 (1994).

Reactive oxygen intermediates (ROIs) have been found to be the messengers in the activation of the κB transcription regulator in mitogen- or cytokine-stimulated cells, operating in conjunction with or independently of various other mechanisms; these include Ca^{++}-dependent and PKC-dependent cytoplasmic signaling pathways. We have recently reported that low-dose ionizing radiation induces NF-κB in human lymphoblastoid 244B cells. Since ionizing radiation generates free radicals in cells, we have investigated whether the ROIs generated by ionizing radiation induce NF-κB activity, and also whether they do so by a similar mechanism as in cells treated with PMA or H_2O_2. The results not only confirm a previous observation from our laboratory that low-dose ionizing radiation (0.1–2.0 Gy) activates κB transcription factor transiently with a maximal induction at 0.5 Gy exposure, but also demonstrate mechanistically that the activation of NF-κB by low-dose ionizing radiation can be inhibited considerably by the antioxidant *N*-acetyl-L-cysteine, indicating that at least the major part of the activation process is mediated by ROIs. These findings support the idea that ROIs can regulate the κB elements which in turn can serve as response elements for oxidant stress.

INTRODUCTION

Nuclear factor κB (NF-κB)[2] is a pleiotropic transcription factor which is associated with the regulation of several genes that code for cytokines and other proteins involved in infection and inflammatory processes (*1–3*). Activation of κB transcription regulator and its translocation into the nucleus represents a rapid response to extracellular signals (*1–3*).

Its expression in cells activated by various mitogens [phorbol 12-myristate 13-acetate (PMA), bacterial lipopolysaccharides (LPS) and lectins] (*4, 5*), cytokines (TNF-α, TNF-β and interleukin-1) (*6, 7*), protein synthesis inhibitors (*8*), calcium ionophores (*9, 10*) and viral infections (*11, 12*) have been studied extensively; more than one pathway may be activated by any single agent. Transient activation of the DNA-binding activity of NF-κB has been reported after exposure *in vitro* to 2–50 Gy of γ or X rays (*13*).

Ionizing radiation at these high doses appears to stimulate NF-κB by several different mechanisms. The transcription of immediate early-response genes that encode transcriptional factors after exposure to ionizing radiation was shown to be mediated by activation of a protein kinase C (PKC)-dependent cytoplasmic signaling pathway (*14, 15*). Tyrosine phosphorylation was reported to be one of the mandatory proximal steps in radiation-induced activation of PKC in cells of a human B-lymphocyte precursor cell line (*16, 17*). However, radiation-induced signaling events which cause the activation of NF-κB and its translocation into the nucleus, as well as activation of other nuclear factors, still remain unclear and require further evaluation.

Treatment of cells with ionizing radiation generates reactive oxygen intermediates (ROIs) which damage DNA and other molecules via indirect action, and which can act as second messengers in intracellular signaling, resulting in the induction of many stress-response genes (*18, 19*). Reactive oxygen intermediates are known to function as a direct mediator in the cytolytic effects of exposure to ionizing radiation (*20, 21*). Recently, it has been observed that activation of NF-κB by other stress-promoting agents, such as H_2O_2, is post-translational; it has been shown to be mediated by ROI-activated PKC (*22*). Other studies have shown the involvement of ROIs in the induction of transcription of other genes, such as c-*jun*, by ionizing radiation (*18*). These

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[2]Abbreviations used: EBV, Epstein-Barr virus; EMSA, electrophoretic mobility-shift assay; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor κB; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonylfluoride; ROIs, reactive oxygen intermediates.

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authors suggested that ROIs induce c-*jun* expression through a protein kinase-dependent mechanism. It is not clear whether ROIs generated by ionizing radiation act post-translationally or to induce mRNA transcription, or even if the ROIs due to ionizing radiation act in a way comparable to that of phorbol esters or hydrogen peroxide (H_2O_2) in the induction of NF-κB activity. The latter question is explored in this study.

Using cells of an EBV-transformed lymphoblastoid cell line, we have confirmed prior observations made in our laboratory (*23*) that exposure to low-dose ionizing radiation (0.1–2.0 Gy), after which cell viability remains high, can induce NF-κB DNA-binding activity, which previously has been reported only after high doses (*13*). The maximum induction was observed 8 h after exposure. Our examination of the mechanism of activation of NF-κB by low-dose ionizing radiation has shown that it can be inhibited efficiently by the antioxidant *N*-acetyl-L-cysteine (NAC), indicating that a major part of the induction was mediated through production of ROIs.

MATERIALS AND METHODS

Cell Culture

EBV-transformed 244B human lymphoblastoid cells (originally supplied by Dr. J. Schwartz) were cultured in complete RPMI-1640 medium containing 2 m*M* L-glutamine and 10 m*M* HEPES (Mediatech, Inc., Herndon, VA) and supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 50 μg/ml gentamicin. The medium did not contain iron salts, which are known to promote the decomposition of H_2O_2 into hydroxyl radicals. The cells were subcultured twice each week with dilution to 2.5×10^5 cells/ml. For all experiments the cells were grown to a maximum cell density of approximately 1×10^6 cells/ml on the day of the experiment.

Exposure to Ionizing Radiation

For each experiment the cells were transferred to fresh complete medium containing 5% FBS with adjustment to a cell density of 1×10^6 cells/ml. The cells were preincubated in this fresh medium for 2 h at 37°C in a humidified incubator with 5% CO_2/95% air in T-75 flasks (Corning, Corning, NY) prior to exposure to ionizing radiation. The exponentially growing cells, immediately after removal from the 37°C incubator, were exposed to doses ranging from 0.1 to 2.0 Gy of ^{137}Cs γ rays at a dose rate of 1.17 Gy/min at room temperature. The irradiations were performed in an Atomic Energy of Canada Ltd. GammaCell-40 Irradiator. Immediately after exposure, the cultures were returned to the 37°C incubator and harvested at 2, 4, 8 and 24 h. Mock-irradiated control cells (0 Gy) were treated identically.

Other Treatments

PMA stimulation. Cells prepared as described above for exposure to ionizing radiation were treated with 50 ng/ml PMA (Sigma) and incubated at 37°C for 2, 4, 8 and 24 h.

Treatment with H_2O_2 and N-acetyl-L-cysteine (NAC). Cells prepared similarly were incubated with 150 μ*M* H_2O_2 (Sigma) or 30 m*M* NAC (Sigma) for 8 h and harvested for electrophoretic mobility-shift assay (EMSA). At these selected concentrations, NAC and H_2O_2 are known to inhibit or induce (respectively) NF-κB activation in other cell lines effectively (*22, 24*). In experiments where the role of ROIs was investigated, the cells were pretreated for 1 h with NAC (30 m*M*) prior to exposure to ionizing radiation or H_2O_2 treatment. The stock solution of 1 *M* NAC was prepared by dissolving NAC in RPMI 1640 medium and adjusting the pH to 7.4 with 3*N* NaOH before addition to the cells.

Nuclear Extract

Nuclear extracts were prepared following the method described by Hillman *et al.* (*25*). Cells which were harvested and then washed in 1× phosphate-buffered saline (PBS) were allowed to swell in buffer A [10 m*M* Hepes, pH 7.8, 10 m*M* KCl, 2 m*M* $MgCl_2$, 1 m*M* dithiothrietol (DTT), 0.1 m*M* EDTA, 0.1 m*M* phenylmethylsulfonylfluoride (PMSF), 1 μg/ml antipain, 0.3 μg/ml leupeptin] for 15 min on ice and pelleted for 3 min at 3000 rpm (800*g*) in a microfuge. The cells were then lysed in buffer A with 0.2% Nonidet P-40. Nuclei were collected by centrifugation as above and resuspended in buffer B [50 m*M* Hepes, pH 7.8, 50 m*M* KCl, 300 m*M* NaCl, 0.1 m*M* EDTA, 1 m*M* DTT, 0.1 m*M* PMSF, 10% (vol/vol) glycerol]. The tubes were allowed to sit in ice for 15 min with occasional mixing; they were then centrifuged for 15 min at 14,000 rpm (16,000*g*) in the microfuge at 4°C. Protein samples were transferred to fresh tubes and stored at –70°C. Protein concentrations were determined using the bicinchonic acid (BCA) method following the manufacturer's protocol (Pierce, Rockford, IL). Fluorescence was measured with an ELISA plate reader (Dynatech MR5000, Chantilly, VA).

Electrophoretic Mobility-Shift Assay (EMSA)

The EMSA was performed using an NF-κB binding protein detection system following the manufacturer's protocol (Promega, Madison, WI) with slight modification. Briefly, a double-stranded oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') containing a tandem repeat of the consensus sequences of (GGG GAC TTT CC) was end-labeled with T_4 polynucleotide kinase. The binding reaction was performed by mixing 15 μg of nuclear extract, 2 μg of poly (dI-dC) (Pharmacia Fine Chemicals, Nutley, NJ) and 111 TBq/mmol [γ-^{32}P]ATP-labeled oligonucleotide probe in binding buffer (10 m*M* Tris, pH 7.5, 50 m*M* NaCl, 1m*M* EDTA, 1 m*M* DTT, 5% glycerol) and then incubating for 20 min at 22°C. For the competition assay the nuclear extract (15 μg) was preincubated with homologous unlabeled NF-κB oligonucleotide for 5 min on ice, followed by addition of [γ-^{32}P]-labeled NF-κB probe. All samples were subsequently electrophoresed at 150 V through 4% polyacrylamide gel in Tris-glycine buffer (25 m*M* Tris, 0.19 *M* glycine and 1 m*M* EDTA). The gels were dried and autoradiographed at –70°C with intensifying screens on hyperfilm (Amersham, Arlington Heights, IL).

Quantitative Analysis

The NF-κB activity in the nuclear extract was determined by quantitative analysis of the dried gel after EMSA using a Betascope 603 Blot Analyzer (Betagen, A Division of Intelligenetics, Inc., Mountain View, CA).

RESULTS

The induction of NF-κB was examined in 244B human lymphoblastoid cells after low doses of ionizing radiation. While viability had decreased at 48 h to 90% after 1 Gy and 59% after 2 Gy, the studies performed here at 8 h at these and lower doses involved predominantly viable cells (Fig. 1A).

To test the effect of low doses of ionizing radiation on the activation of NF-κB, the cells were irradiated with 0.1, 0.5, 1.0 or 2 Gy, incubated for 8 h after exposure and harvested. The induction of DNA-binding activity of NF-κB in the nuclear extract of the treated cells was analyzed by EMSA with [γ-^{32}P]-labeled κB specific DNA probe. The

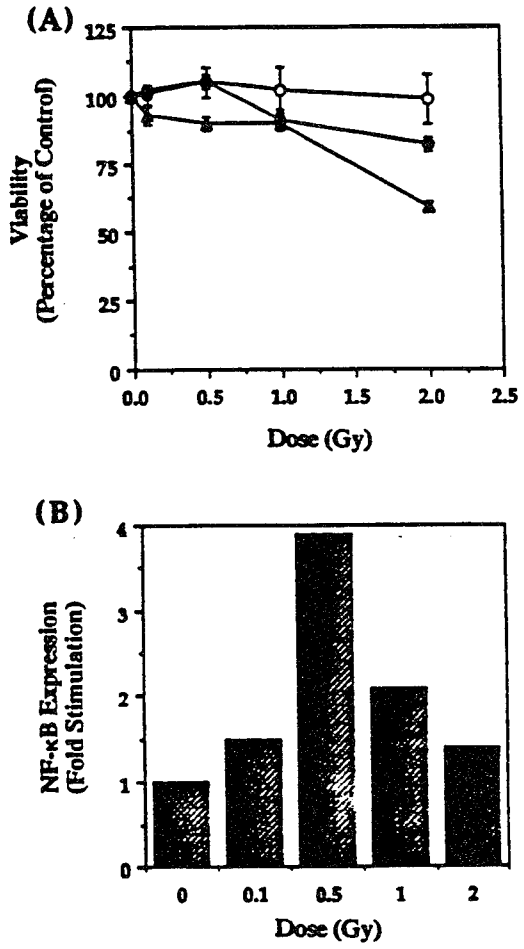

FIG. 1. Panel A: Effect of low-dose ionizing radiation on the viability of 244B cells. Viability of 244B cells was measured by the trypan blue dye exclusion method at (○) 8, (●) 24 and (▲) 48 h after irradiation. Each point is the arithmetic mean ± SEM of two cell counts from each of two independent treatment flasks. The results are plotted as a percentage of control (0 Gy) viability. Panel B: Dose-dependent activation of NF-κB by low-dose ionizing radiation. Equal amounts of protein (15 μg) from nuclear extract isolated from 244B cells after 8 h in culture after different doses of irradiation (0.1–2.0 Gy) were subjected to EMSA analysis and quantification (See Materials and Methods).

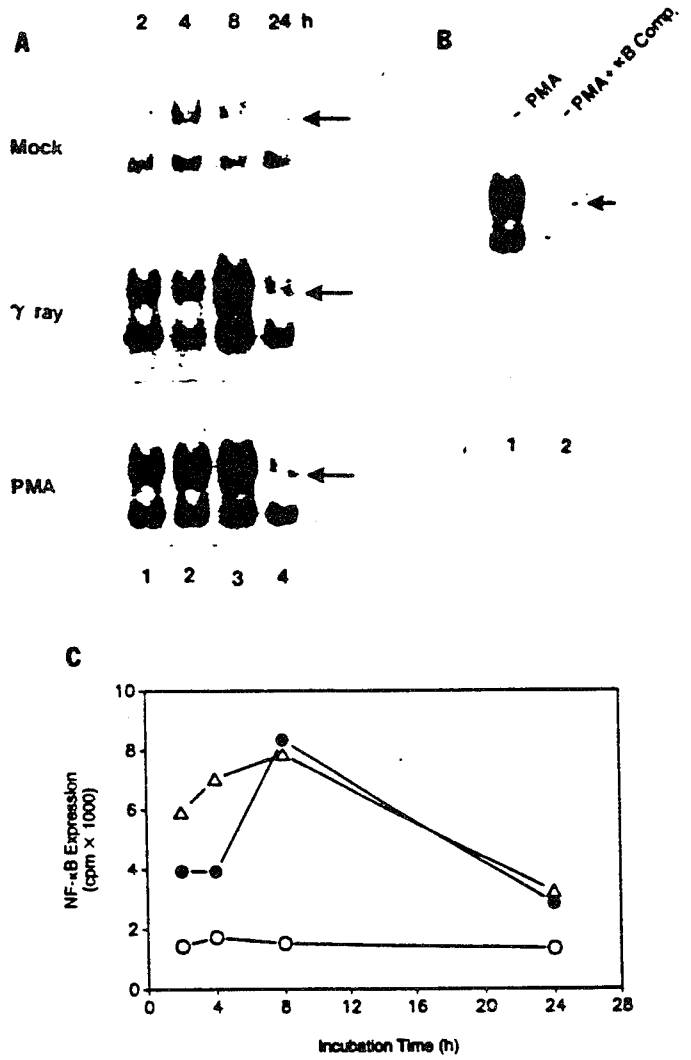

FIG. 2. Panel A: Time dependence of the NF-κB activity in 244B cells after 0.5 Gy exposure or PMA treatment. Equal amounts of protein (15 μg) from nuclear extracts were analyzed by EMSA as described in Materials and Methods. Lanes 1, 2, 3 and 4 show cells harvested at 2, 4, 8 and 24 h of incubation, respectively, from mock-irradiated, γ-irradiated (0.5 Gy) and PMA-treated (50 ng/ml) cultures. The autoradiogram of a representative native gel is shown. Complexes tested for specific binding of NF-κB are indicated by the arrow. Panel B: Nuclear extract derived from the cells stimulated with 50 ng/ml PMA and harvested after 8 h. Nuclear extract (15 μg) was preincubated in the absence (lane 1) or presence (lane 2) of 250 molar excess of homologous unlabeled NF-κB-specific double-stranded oligonucleotide competitor for 5 min on ice followed by the addition of [γ-^{32}P]-labeled NF-κB specific probe. Complexes tested for specific binding of NF-κB are shown by the arrow. Panel C: Betascope blot quantification of the dried gel of the autoradiogram shown in Fig. 2A. The data shown represent the counts per minute of incorporated [γ-^{32}P]ATP.

NF-κB-specific band was quantified. Induction of NF-κB was observed at the lowest dose of 0.1 Gy, with a maximum of induction at 0.5 Gy. As the exposure was increased to 1.0 Gy, the induction decreased, almost reaching the constitutive level of the mock-irradiated control after the 2-Gy exposure. The activation was 1.5-, 3.9-, 2.1- and 1.4-fold (compared to the control level) after exposures of 0.1, 0.5, 1.0 and 2.0 Gy, respectively (Fig. 1B).

To determine whether the induction of NF-κB after 0.5 Gy was transient or persistent, the 244B cells were irradiated and harvested for EMSA at 2, 4, 8 and 24 h after exposure. The results observed were therefore for cells within one cell cycle time after exposure. A time-dependent increase in NF-κB binding activity was observed (Fig. 2A). At 2 and 4 h after exposure, the increase compared to mock-irradiated cells was 2.8- and 2.3-fold (Fig. 2C): there

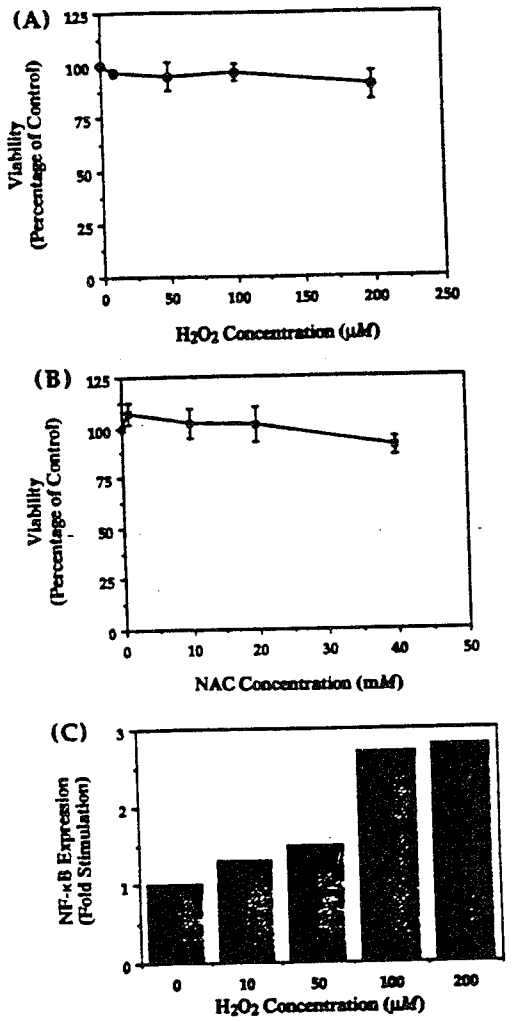

FIG. 3. Panel A: Viability of 244B cells in the absence or presence of various concentrations of H_2O_2. The number of viable cells was determined after 8 h in culture by trypan blue dye exclusion. Each point is the arithmetic mean ± SEM of two cell counts from two independent treatment flasks and is expressed as a percentage of the viability of untreated control cells. Panel B: Viability of 244B cells in the absence or presence of various concentrations of NAC after 8 h in culture. Each point is the arithmetic mean ± SEM of two cell counts from two independent treatment flasks and is expressed as a percentage of viability of untreated control cells. Panel C: Dose-dependent activation of NF-κB by H_2O_2. Equal amounts of protein (15 μg) from nuclear extracts isolated from 244B cells after 8 h in culture at various concentrations of H_2O_2 (10–200 μ*M*) were subjected to EMSA analysis (see Materials and Methods). Total amount of NF-κB activation in nuclei was determined by quantitative analysis of the dried gel using a Betascope blot analyzer.

was then a considerable increase (5.5-fold) to a maximum induction at 8 h. At 24 h after exposure, the DNA-binding activity was reduced toward levels seen in unexposed samples (2.1-fold). In contrast to the radiation response, in PMA-treated cells (Fig. 2A), considerable increases occurred at 2 and 4 h (Fig. 2C); the NF-κB induction was 4.1-fold at 2 h and 4.1-fold at 4 h. At 8 h the induction again appeared to be at a maximum (5.2-fold) and as after radiation exposure was reduced at 24 h toward the control level (2.4-fold). As confirmation that we were restricting the quantitative analysis to the NF-κB-specific region of the gel, competition experiments were performed. Nuclear extracts (derived from PMA-stimulated 244B cells) preincubated with 250-fold molar excess of unlabeled oligonucleotide encompassing the NF-κB binding motif followed by the addition of [γ-^{32}P]-labeled NF-κB probe, confirmed the κB-specific binding (Fig. 2B).

For comparison to the response to ionizing radiation, the involvement of the reactive oxygen intermediate (ROI) pathway in the expression of DNA-binding activity at 8 h after initiation of an exposure to H_2O_2 was also examined. The effect of H_2O_2 and NAC treatments on the viability of 244B cells at concentrations of 10–200 μm and 1–40 m*M*, respectively, was determined. The viability was greater than 97% at 8 h of incubation for concentrations up to 100 μm of H_2O_2 (Fig. 3A). At 200 μ*M*, the viability was decreased only slightly at 8 h (90%). When the cultures were grown in the presence of NAC at final concentrations of 1–40 m*M*, no toxicity was observed at concentrations up to 20 m*M*; a slight decrease in cell viability (90% survival) was observed at 40 m*M* final concentration (Fig. 3B).

For examining the effect of H_2O_2 on the induction of NF-κB, cells were treated with H_2O_2 at final concentrations of 10, 50, 100 and 200 μ*M* and were harvested at 8 h. The DNA-binding activity was determined quantitatively by EMSA and the gels were analyzed with a Betascope analyzer. The H_2O_2-activated DNA-binding activities of NF-κB were 1.3-, 1.5-, 2.7- and 2.8-fold at these concentrations, respectively (Fig. 3C).

Investigation of the Role of the ROI Pathway in Expression of NF-κB Induced by Low-Dose Ionizing Radiation and H_2O_2

For this study, the 244B cells were exposed to 0.5 Gy and harvested at 8 h. In a parallel culture the cells were pretreated with 30 m*M* NAC for 1 h, exposed to 0.5 Gy, reincubated and harvested 7 h later. This experiment was repeated on three separate occasions. Treatment of the 244B cells with 0.5 Gy alone again demonstrated a profound increase in the induction of NF-κB DNA-binding activity (lane 3, Fig. 4). The results of the three experiments were then averaged. The response for the specific NF-κB band was a 6.0 ± 1.7-fold increase compared to mock-irradiated control cells (Table I). When the irradiated cells were treated with the antioxidant NAC for 1 h before exposure, a significant decrease in the DNA-binding activity of NF-κB was observed (lane 5, Fig. 4). The Betascope analysis, averaged for the three experiments, showed a 58% decrease in DNA-binding activity (to a 2.7 ± 0.4-fold induction level) compared to 0.5 Gy alone.

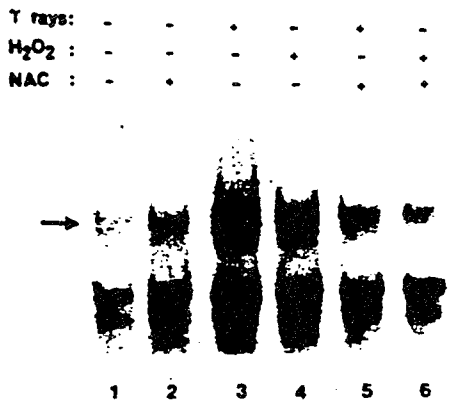

FIG. 4. Inhibitory effect of NAC on the activation of NF-κB by 0.5 Gy irradiation or H_2O_2 treatment. 244B cells either irradiated with a total dose of 0.5 Gy or treated with 150 μ*M* H_2O_2 were cultured for 8 h. When NAC pretreatment was studied, the incubation time after irradiation or with H_2O_2 treatment was 7 h. Equal amounts of protein (15 μg) from nuclear extracts were incubated with [γ-^{32}P]ATP-labeled NF-κB specific double-stranded-oligonucleotide probe in binding buffer and subjected to EMSA analysis and quantification. The autoradiogram of the native gel is shown. Complexes tested for specific binding are indicated by the arrow.

TABLE I
The Inhibitory Effect of Antioxidant NAC on the Activation of NF-κB by Low-Dose Ionizing Radiation or H_2O_2

Treatment/exposure	NF-κB activation	
	cpm (1 × 10^6)[a]	Fold stimulation[b]
Mock-irradiated	0.35 ± 0.12 (0.24–0.55)[c]	—
NAC (30 m*M*)	0.4 ± 0.08 (0.39–0.61)	1.4 ± 0.19 (1.1–1.6)
0.5 Gy	2.05 ± 0.63 (1.05–2.78)	6.0 ± 1.70 (4.3–8.7)
H_2O_2 (150 μM)	1.72 ± 0.43 (1.12–2.10)	5.2 ± 1.60 (3.8–7.7)
0.5 Gy + NAC (30 m*M*)	0.89 ± 0.16 (0.74–1.15)	2.7 ± 0.42 (2.1–3.3)
H_2O_2 (150 μ*M*) + NAC (30 m*M*)	0.83 ± 0.02 (0.79–0.84)	2.7 ± 0.71 (1.5–3.4)

[a]Each value is the mean ± SEM of three independent experiments.
[b]Each value is the mean ± SEM of the fold stimulations (the increase above the level found in the mock-irradiated controls) from the three independent experiments.
[c]Values in parentheses indicate the range.

To study the role of ROIs during H_2O_2 induction of NF-κB activity, 244B cells were treated with 150 μ*M* H_2O_2 for 8 h, while in a parallel culture the cells were pretreated with 30 m*M* NAC for 1 h and then treated with 150 μ*M* H_2O_2 for 7 h. The cells were then harvested for EMSA analysis. Treatment of 244B cells with H_2O_2 alone, at this concentration and for this treatment time, resulted in an increased level of NF-κB binding activity similar to that observed with cells exposed to 0.5 Gy at 8 h after exposure. The response, averaged for the three experiments, was a 5.2 ± 1.6-fold increase compared to untreated control cells (Table I). The cells pretreated with NAC for 1 h before H_2O_2 treatment showed a considerable decrease in the DNA-binding activity of NF-κB (lane 6, Fig. 4). The Betascope analysis revealed a 48% decrease in DNA-binding activity (to a 2.7 ± 0.7-fold induction level) compared to cells treated with H_2O_2 alone (Table I). In the cells treated only with the antioxidant NAC, there was little if any effect on the appearance of DNA-binding activity of NF-κB (lane 2, Fig. 4; Table I). For cells either exposed to 0.5 Gy or treated with H_2O_2 in the presence of NAC, although considerable reductions in the expression of NF-κB were observed, the DNA-binding activity was not reduced to the constitutive level. While there was a 1-h difference in the incubation period after radiation or H_2O_2 treatment for cells incubated with and without NAC, there is little likelihood that the large percentage reductions observed with the NAC present can be attributed to the difference between the 7- and 8-h incubation times.

DISCUSSION

Activation of NF-κB by Low-Dose Ionizing Radiation

We reported for the first time (*23*) and have confirmed in this study that exposure of cells of a mammalian cell line derived from the human immune system to low-dose ionizing radiation can cause a transient increase in the DNA-binding activity of NF-κB with increasing incubation times after exposure: the increase was maximal after 0.5 Gy at 8 h. We are also reporting here for the first time that this induction of NF-κB by low doses of ionizing radiation, where cell viability remains high, is mediated through reactive oxygen intermediates. The activation of NF-κB was efficiently (although not completely) inhibited by the antioxidant NAC.

Recently, exposures to high doses (5–50 Gy) of ionizing radiation were reported to induce the expression and DNA-binding activity of NF-κB in KG-1 cells (*13*). Other studies (*14*, *18*, *26*) also indicated that high doses of ionizing radiation (2–50 Gy) induce transient expression of many genes and gene products. However, in many cell types these high doses are likely to result in extensive DNA damage and cell killing (*13*, *27*). In contrast to these studies of NF-κB induction after high radiation doses, it has been demonstrated here that low doses of ionizing radiation (0.1–2.0 Gy), in a biologically more relevant dose range where cell viability remains high (close to 97%), can induce a marked increase in DNA-binding activity of NF-κB in 244B cells. This is important since these genes and gene products are in turn involved in the induction of many distinct classes of transcription factors.

Also of possible importance is our observation that the maximum expression of NF-κB after low-dose ionizing radiation occurred at a later time after exposure than was reported previously by other laboratories after high-dose exposure (*13*). At a low dose of 0.5 Gy we observed a maximum induction of DNA-binding activity of NF-κB at 8 h, with the activity approaching the constitutive level at 24 h. After a high dose (10 Gy) of ionizing radiation, the DNA-binding activity in EBV-transformed lymphoblastoid (C3ABR and C5ABR) cells was previously shown to reach a maximum at 1 h, with the activity disappearing from the nuclei at 9 h after irradiation (*28*). In human KG-1 myeloid leukemia cells, NF-κB expression and binding activity were shown to reach a maximum at 2–4 h after exposure to a dose of 20 Gy; this declined to the pretreatment level by 12 h (*13*). The time frame reported here after low doses of ionizing radiation is similar to that found in UV-irradiated HeLa Tk^- cells (*29*) or PMA-treated primary human T cells (*30*); the maximal induction was also shown at 8 h. These findings allow for the possibility that different mechanisms are involved at different levels of exposure to ionizing radiation, with resulting biological outcomes which are different. Alternatively, this shift in time at which a maximum induction of NF-κB is observed could depend on the type of cell. Mechanistically, different signaling pathways may be involved in the different dose ranges. With regard to biological outcomes, after exposure to low-dose ionizing radiation the activation of NF-κB could be a rapid response serving as a protective measure against radiation insult or injury to the cells, or a communication path to other cells. The activation of NF-κB, which is a pleiotropic transcription factor, could regulate the expression of genes that code for many gene products supportive of cell survival, thereby circumventing the longer-term cellular damage which would otherwise be induced only after high doses of ionizing radiation. In the hypothesized communication role, the induction could be similar to that occurring in infection and inflammatory processes. The issue of whether the activation of NF-κB binding activity by low-dose ionizing radiation is associated with either an increase in expression of mRNA for the NF-κB, or is occurring subsequent to the activation of pre-existing protein by dissociation of IκB from NF-κB and mobilization of the latter into the nucleus (or both), remains to be determined. The possibility of the former is suggested by the report of a detectable increase in NF-κB mRNA expression after exposure to high-dose ionizing radiation at 20 Gy (*13*).

Activation of NF-κB is mediated through ROIs. NF-κB induced by high-dose ionizing radiation has been shown to be associated with the activation of a PKC-dependent cytoplasmic signaling pathway (*16*). Tyrosine phosphorylation was also found to be one of the mandatory steps in radiation-induced activation of a PKC in human B-lymphocyte precursors (*16*). In addition to these signaling pathways, in cells stimulated by mitogens (PMA and LPS) or cytokines (TNF-α and IL-1), another signaling pathway involves ROIs. This has been shown to play an important role in the activation of NF-κB (*18*). Further evidence for the involvement of ROIs in the induction of NF-κB came from recent work where the antioxidants NAC or L-cysteine were shown to block NF-κB by scavenging free radicals (*24*). Since ionizing radiation is known to induce ROIs, we investigated, using the antioxidant NAC, the involvement of the ROI signaling pathway in the induction of NF-κB binding activity induced by low-dose ionizing radiation. The pretreatment of 244B cells with NAC at a concentration of 30 m*M* inhibited more than 55% of the activation of NF-κB induced by 0.5 Gy. This finding strongly supports the hypothesis that oxygen radicals play an important role in the activation of NF-κB. The lack of complete suppression of NF-κB by NAC may indicate either that the activation of NF-κB by ionizing radiation involves more than one signaling pathway or, alternatively, that NAC may not be 100% effective (*31*).

The effect of low-dose ionizing radiation on the induction of the DNA-binding activity mediated through ROIs may be attributed to different mechanisms. The activation of NF-κB could be mediated by an intranuclear signaling cascade. The free radicals produced by ionizing radiation cause DNA damage which can eventually result in a reverse signaling mechanism from the nucleus to the cytoplasm as a cellular response (*13*). This response possibly involves activation of PKC, resulting in translocation of NF-κB into the nucleus. A number of DNA-damaging agents, such as 1-β-D-arabinofuranosylcytosine (ara-C), UV light, alkylating agents and etoposide, have been reported to induce c-*jun* transcription (*32–35*), which is known to occur as a result of activation of PKC (*34*).

Alternatively, the signaling cascade leading to the activation of NF-κB by low-dose ionizing radiation may be initiated at the plasma membrane, as has recently been reported in UV-irradiated enucleated HeLa S3 cells (*36*). Oxidants such as H_2O_2 and superoxide, produced by ionizing radiation through radiolysis of bound and solvent water in the cell, can induce the activation of PLC-γ, leading to mobilization of Ca^{++} and activation of PKC (*18, 37*). The transient activation of PKC by ROIs (*18*) produced by ionizing radiation, or the direct action of the ROIs produced by ionizing radiation, may cause the release of IκB from the p50-p65-IκB complex, followed by increased nuclear localization of NF-κB. The intracellular ROIs in Jurkat T cells generated by the addition of micromolar concentrations of H_2O_2 were shown to activate NF-κB by inducing the release of the inhibitory subunit IκB (*22*). Taken together, these findings indicate that the DNA-binding activity of NF-κB induced by low-dose ionizing radiation described here may involve more than one signaling pathway.

although the major part of the induction is likely to be mediated through production of ROIs. Thus the present findings are consistent with the concept that κB elements in regulatory domains of genes are involved in the cellular response upon oxidant stress (*19*).

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INDUCTION OF "IMMEDIATE EARLY GENES" BY LOW DOSE IONIZING RADIATION

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ABSTRACT

The induction of immediate early genes (IEGs) by ionizing radiation has been previously reported after high doses, in the range of 5-50 Gy, delivered at high dose rates (above 14 Gy/min). In contrast, the present study reports the induction, in an EBV transformed human lymphoblastoid cell line (244B), of the protooncogenes c-*fos*, c-*jun*, c-*myc* and c-*Ha-ras* after low doses of ionizing radiation, in the range of 0.25-2 Gy, and at a more standard dose rate of 1.17 Gy/min. Time course studies demonstrated that all four IEGs are maximally induced at 1 h post-radiation exposure, with the maximum response in this dose range studied occurring after 0.5 Gy for all but c-*fos*, which showed a maximum response after 0.25 Gy. Using various second messenger signalling inhibitors, an initial investigation of the pathways involved was undertaken. All four of the IEGs induced by low dose (0.5 Gy) radiation shared a common pathway of tyrosine kinase activation, similar to a pathway associated with PMA induction in these cells. Other signalling events are also involved, but these have been shown, using selected inhibitors, to be specific to the different protooncogenes studied. Differences in induction pathways for three of the four protooncogenes have also been observed for PMA versus 0.5 Gy radiation.

INTRODUCTION

Studies of the biological responses to ionizing radiation are extremely diverse; these often focus on events after high doses, both early and late occurring, which can be related to cell killing. Cellular and molecular responses after low doses are relatively unexplored. The underlying mechanisms for many phenomena, e.g. cancer induction, are still not completely understood. While radiation can cause damage to all classes of biological molecules, investigations of the effects of radiation on RNA synthesis in mammalian cells have generally been found to be unaffected or to be repressed shortly after high doses of radiation [1]. In a study looking for quantitative and qualitative changes in the RNA synthesized immediately after radiation, using an early RNA/DNA hybridization technique, high doses (e.g. 25 Gy) were also used [1].

Recent studies have demonstrated that certain cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and immediate early genes (IEGs) such as the c-*fos*/c-*jun* family, c-*myc* and c-*Ha-ras*, are induced by ionizing radiation in different cell types after high doses, distinguishing these studies from the earlier reports of RNA synthesis inhibition after high doses. The induction was reported to be mediated through protein kinase C (PKC) and reactive oxygen intermediate (ROI) pathways [2-5]. In all of these studies, the induction was examined after exposure of the cells to high doses, ranging from 5 - 50 Gy. The dose rates in those studies were at or above 12 Gy/min. Such high doses would likely result in prolonged mitotic inhibition, cell cycle progression delay, and extensive cell death [6-8]. In a much lower dose range, 0.5 - 2.5 Gy, there are two reports of induction of gene expression. One shows induction of $\alpha||b\beta 3$ integrin in melanoma cells after an exposure of 0.5 Gy [8], and a second shows induction of prostaglandins after doses of less than 2 Gy [9]. The expression of IEGs, however, has not been previously reported after similar low doses of ionizing radiation (less than 2 Gy).

The total number of IEGs are thought to be close to 100, but only a few have been fully characterized. The induction of four of these genes, c-*fos*,

c-*jun*, c-*myc* and c-*Ha-ras* is rapid and transient in many cells and tissues in response to various stimuli [10-12]. One mechanism of induction of these four IEGs involves the PKC activation pathway [5,13-17] and requires no new protein synthesis. Because of the importance of these regulatory genes, we have investigated the effect of low dose ionizing radiation (0.25 - 2 Gy) on their expression in a cell line representative of the human immune system, the Epstein Barr Virus (EBV) transformed 244B human lymphoblastoid cell. In relation to this induction, we have also examined the role of several possible cellular signalling pathways.

Methods and Materials

Cell culture and irradiation

EBV transformed 244B human lymphoblastoid cells (originally supplied to our laboratory by Dr. J. Schwartz) were regularly maintained in RPMI-1640 medium containing 2 mM L-glutamine and 10 mM HEPES (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 50 μg/ml gentamicin (Sigma, St. Louis, MO). The cells used in each experiment were always collected at 72 h after initiation of the cell culture, when they had achieved a density of approximately $1x10^6$ cells per ml. Cell viability was determined using the trypan blue dye exclusion method at the start of each experiment; it was always ≥98%. Prior to each experiment, the cells were washed, counted (Coulter Electronics, Inc., Hialeah, FL) and transferred to fresh complete medium. The cell density was always adjusted to $1x10^6$ cells/ml in a final volume of 5 ml in T-25 flasks (Corning, Corning, NY) at the time of induction. After resuspension, the cells were incubated for 30 min at 37°C in a humidified incubator with an atmosphere of 5% CO_2/95% air. Chemical induction of IEGs was then initiated with the addition of 20 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma), and the cells were incubated for various pre-determined time periods (0-16 h). In the irradiation studies, after a 30 min preincubation in fresh medium, the cells were exposed to various doses of ^{137}Cs γ rays (0.25-2Gy) at a dose rate of 1.17 Gy/min at room temperature in an Atomic Energy of Canada

GammaCell-40 Irradiator and immediately reincubated at 37°C. Appropriate mock irradiated controls without PMA and radiation exposure were simultaneously incubated. After incubation, the cells were harvested by centrifugation (Beckman, Model TJ-6) for 5 min at 250 x g and resuspended in phosphate buffered saline (PBS); they were then washed twice with PBS in a microcentrifuge (Eppendorf, Model 5415C, Rotor S-45-18-11, Westbury, NY) at 14,000 rpm. The resulting pellet was quick frozen in liquid nitrogen and stored at -70°C.

Second messenger inhibitors

In studies of signal transduction pathways, various second messenger inhibitors were added to the flasks 30 min prior to the addition of 20 ng/ml PMA or exposure to 0.5 Gy ^{137}Cs γ-rays. The flasks were then incubated for 1 h at 37°C after PMA addition or radiation exposure. The inhibitors used were nifedipine, a calcium channel blocker (50 μm); N-acetylcysteine, a scavenger of ROIs (30 mM) (Sigma, St. Louis, MO); calphostin C, an inhibitor of PKC (50 nM) and genistein (2.6 μm), an inhibitor of tyrosine - specific kinase (protein tyrosine kinase: PTK). The concentrations used for each of these inhibitors was equivalent to the reported IC50 concentrations (Calbiochem, LaJolla, CA) [18].

RNA isolation and Northern blot analysis

Total cellular RNA was isolated by the acid guanidinium isothiocyanate-phenol-chloroform extraction method [19]. The RNA was quantified spectrophotometrically at 260 nm (the ratio of A_{260} nm to A_{280} nm always exceeded 1.8), and aliquots of 20 μg of total cellular RNA were size fractionated by electrophoresis on a 0.8% agarose/2.2 M formaldehyde gel. The RNA was then electroblotted from the gel using 0.025 M phosphate buffer (pH 6.5) onto Gene Screen Membrane (New England Nuclear, Boston, MA) and immobilized by UV cross-linking [Stratagene, CA]. The RNA was then prehybridized for at least 4 h at 42°C in a buffer containing 50% formamide, 0.1% sodium dodecylsulfate (SDS), 5xSSC (standard saline citrate), 2.5 x Denhardt's solution, 250 μg/ml salmon sperm DNA, and 50 mM Na_2PO_4, pH 6.5. The membranes were then hybridized for at least 16 h at 42°C

with specific radiolabeled cDNA probes in the same prehybridization buffer, supplemented with 10% dextran sulfate. At the end of hybridization, each membrane was washed stringently with 6 x SSPE (sodium chloride, sodium phosphate, EDTA) containing 0.5 % SDS at room temperature for 5 min (two times), 1 x SSPE containing 0.5% SDS at 55°C for 10 min and 0.1 x SSPE containing 0.5% SDS at 55°C for 10 min. The membranes were autoradiographed at -80°C on X-ray film (X-Omat AR, Eastman Kodak) with intensifying screens for three days.

cDNA probes: cDNA probes used in this study were as follows: (a) *c-fos*, a 9.0 κb ECO RI fragment; (b) *c-jun*, a 2.6 kb EcoRl fragment; (c) *c-Ha-ras*, a 3.0 kb Bam Hl fragment; and (d) *c-myc*, a 1.4 kb EcoRl fragment (Oncor, Gaithersburg, MD). GAPDH (glyceraldehyde-3-phosphate dehydrogenase), a 1.2 kb pst-1 fragment (Boehringer Mannheim) was used as an internal housekeeping control gene. The latter is constitutively expressed in most tissues and is the most widely accepted internal control for assessing RNA loading and transfer. Random-primed labeling was performed with ^{32}P-dCTP (~3000 Ci/mmol, Amersham Arlington Heights, IL), yielding probes with a specific activity of ~ 1 x 10^9 cpm/μg.

Densitometric Analysis

Autoradiograms were analyzed using a desktop digital imaging method [20]. Briefly, the autoradiograms were photo-screened by standard video imaging equipment connected to a Macintosh computer (Model IIci) and the image was captured onto a 2HD Sony computer diskette using an integrated density program (NIH 1.45 image analysis software). The image was analyzed using the same program. The area to be quantitated for each band was kept constant for all of the bands in an autoradiogram. Background density on an autoradiogram was substracted from the densitometric data of each band.

RESULTS

Other recent studies from our laboratory [21] have demonstrated that, in the low dose range of 0.10 Gy to 2.0 Gy, a maximum induction of the DNA binding protein NF-κB occurred in these same 244B human lymphoblastoid cells after a dose

of 0.5 Gy. This was observed at several different times of incubation post-exposure. We therefore choose this same dose to investigate the induction of the protooncogenes c-*fos*, c-*jun*, c-*myc* and c-*Ha-ras*. The induction was examined as a function of incubation time post-exposure (0.25, 0.5, 1,2,4,8,12 and 16 h). The results are shown in Figure 1. The expression of the housekeeping gene GAPDH, which served as a control, was approximately the same at all of the incubation times studied. The densitometric analysis data of Figure 1, normalized to GAPDH, is presented in Figure 2. The autoradiogram shows that 0.5 Gy of ionizing radiation induces all four proto-oncogenes, and that the maximum induction after this dose occurred at 1 h post-exposure. When compared to their levels at 0 h, the normalized RNA ratios (Figure 2) reveal 189, 10 & 8, 7, and 10-fold increases at 1 h for c-*fos*, c-*jun* 1st and 2nd bands, c-*myc* and c-*Ha-ras*, respectively. For c-*fos*, barely observable induction (which can be seen on the original autoradiogram) appears within 15 min of exposure (Figure 2), and becomes profound at 1 hr. The induction of expression quickly shuts down, as the c-*fos* is only minimally present at 2 hr post-exposure. For c-*jun*, the expression of both transcripts was more obvious at 30 min, again reaching its maximum at 1 h. At 2 hr, the c-*jun* transcripts were reduced, but not to as great an extent as for c-*fos*. The c-*jun* seemed to reappear at 16 hr post-exposure; this was not observed for the c-*fos*. For c-*myc*, the levels at 15 and 30 min followed the same pattern as for c-*fos*, appearing similar to the constitutive level (which was measured immediately post-exposure) at 15 min, with an increase at 30 min, and again a profound increase at 1 hr. The decreased level present at 2 hr showed considerable c-*myc* still present, with this decreasing to the constitutive level at 4 hr. As was the case for the c-*jun*, but more obviously, a second induction of c-*myc* was observed at 16 hr. The time frame of the induction of c-*Ha-ras* was similar to that of c-*jun* and c-*myc* before 1 hr, while the decrease and later increase were similar to c-*myc* after 1 hr.

Since we had observed that 1 h is the post-exposure time when maximum induction occurs after the dose of 0.5 Gy (for the protooncogenes studied), we further investigated the dose-dependence of their induction at 1 h after several

other doses of ionizing radiation (0.25, 0.5, 0.75, 1 and 2 Gy). For comparison, we also examined their induction at 1 hr in PMA treated and untreated cells (the latter is a 0 dose control). The results are presented in Figure 3, and the densitometric analysis data normalized to GAPDH expression is presented in Figure 4. The PMA treated cells exhibited 5, 10 & 14, 12 and 4.5-fold increased expression over the constitutive (-PMA) levels, respectively, for c-*fos*, c-*jun* 1st and 2nd bands, c-*myc* and c-*Ha-ras*. The maximum induction of mRNA expression for c-*jun*, c-*myc* and c-*Ha-ras* occurred after the 0.5 Gy dose; for c-*fos*, however, the maximum was observed after the lower dose of 0.25 Gy. The induction of *c-fos* then decreased in a dose-dependent manner; it was lower at 0.5 Gy, decreased slightly further at 0.75 Gy, while at 1 Gy (and also 2 Gy) it was at its constitutive level. The induction of c-*jun* and c-*myc* followed similar patterns after their maximum at .5 Gy, i.e., lower expression for both after 0.75 Gy, and then decreasing again at 1 Gy, where it remained after 2 Gy for c-*jun*. An increased induction of c-*myc* was again observed after 2 Gy. For c-*Ha-ras* the induction increased from several-fold above the control level after 0.25 Gy to a maximum after 0.5 Gy, and then declined after 0.75 Gy to a level equivalent to the 0.25 Gy value. The constitutive level of expression was observed after 1 Gy, while a second induction, as for the c-*myc*, was observed again after 2 Gy. The normalized RNA level of c-*jun* 1st and 2nd bands, c-*myc* and c-*Ha-ras* at their maximum values after 0.5 Gy was 20 & 26.5, 19, and 7-fold greater than that of the untreated control levels, respectively, while the maximal normalized level for c-*fos* after the 0.25 Gy dose was 12-fold as compared to the constitutive level.

The data presented in the two preceding experiments demonstrated that the maximum level of expression for all four early genes studied occurred at 1 h; that the maximum expression of c-*jun*, c-*myc*, and c-*Ha-ras* occurred after the dose of 0.5 Gy at this time, and that the maximum expression for c-*fos* occurred after the lower dose of 0.25 Gy. The 1 hr time point and 0.5 Gy dose were therefore

selected for use in the subsequent studies investigating the signalling pathway(s) responsible for the protooncogene induction. These studies involved the use of various second messenger inhibitors, including a calcium blocker (nifedipine), a reactive oxygen intermediate scavenger (NAC), a protein kinase C inhibitor (calphostin C), and an inhibitor of tyrosine-specific kinase (genistein). The inhibitors were also used with PMA treated cultures, to compare the pathways involved in the radiation induction to those active in PMA induction. The data is presented in Figure 5. The densitometric analysis data for these studies, normalized to GAPDH, is shown in Figure 6.

The Role of Different Pathways in Gene Induction:

With evidence in hand that low dose ionizing radiation can induce different protooncogenes, and that the dose at which a maximum response can be observed is different for one of the four protooncogenes studied, we examined the possibility that different pathways might contribute to the induction of different protooncogenes. We also explored the possibility that the pathways involved in low dose ionizing radiation induction might not be the same as as after a standard chemical (PMA) induction.

PMA Induction and Pathway of Signal Transduction:

PMA induction of c-*fos* in 244B cells was selectively inhibited by NAC and genistein, showing 56.5 and 47.4% decreases respectively in c-*fos* expression compared to PMA induction alone. Reduction by nifedipine was negligible, and minimal by calphostin C. These results suggest that the c-*fos* expression by PMA is mediated through reactive oxygen intermediates and PTK pathways but not through calcium dependent pathways and only weakly through protein kinase C pathways (Table 1). Unlike c-*fos*, the c-*jun* expression upon PMA induction (both transcripts) involves calcium dependent kinase (nifedipine inhibited, 61.5/50.0%*) and PTK dependent (genistein inhibited, 81.8/83.1%) pathways but not

the PKC pathway, because there is no inhibition by calphostin C. There is some inhibition by NAC (23.1/16.7%), indicating minimal ROI involvement. PMA induction of c-*myc* involved Ca^{++} dependent (nifidipine inhibition, 60%) and tyrosine kinase signalling (geneistin inhibition, 72.2%) pathways. Also for c-*myc*, similar to c-*jun* but in contrast to c-*fos*, ROIs played only a small role in induction (NAC inhibition, 16.7%). For c-*myc* however, PKC seemed to be involved somewhat more in induction (calphostin C inhibition 23.1%) than for any other protooncogene.

For c-*Ha-ras*, in contrast to c-*fos*, c-*jun* and c-*myc*, expression was found to be inhibited only by genestein (41.2%), indicating involvement only of the PTK dependent pathway, and not calcium, reactive oxygen intermediates or PKC dependent pathways.

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* The values (___/___) were first and second transcripts of c-*jun*.

These results indicate that the PTK pathway is the only one involved in the PMA induction of all four IEGs, and the only one active in the PMA induction of c-*Ha-ras*. The calcium channel pathway is not involved in the PMA induction of c-*fos* and c-*Ha-ras*, while it is involved in *c-jun* and *c-myc* induction. The PKC pathway (Table 1) is involved in c-*myc* and weakly in c-*fos* induction, but not in c-*jun* and c-*Ha-ras* induction. ROIs are involved in c-*fos* and c-*myc* induction, weakly in c-*jun* induction, and not in c-*Ha-ras* induction; the PKC pathway is not involved in c-*jun* induction (Table 1).

Ionizing Radiation Induction and Mechanism of Signal Transduction:

For c-*fos*, the induction by 0.5 Gy radiation is inhibited by nifidipine, calphostin C and genistein by 44.4, 52.4 and 44.5%, respectively, indicating that the calcium channel, PKC and PTK pathways are involved. No inhibition was observed with NAC, indicating that (in this cell line) the ROI pathway is not involved. For radiation induction of c-*jun* and c-*myc*, all four pathways examined

are found to be involved; the PTK pathway for both protooncogenes was inhibited to the greatest extent. The radiation induction of both transcripts of c-*jun* by 0.5 Gy was inhibited; by 28.6/23.1, 63.0/63.0, 28.6/37.5, and 78.3/75.6%, respectively, in the presence of nifidipine, NAC, calphostin C and genistein. For c-*Ha-ras*, the decreases were 58.3 in the presence of nifidipine, 23.1% by NAC, 3.8% by calphostin and 66.7% by genistein. Only a small percent of inhibition was observed for calphostin, indicating that the calcium, ROI and PTK pathways are involved, but that the PKC pathway is only minimally involved.

These results indicate that the calcium channel and PTK pathways are considerably involved in immediate early gene induction by ionizing radiation for all four genes. The ROI pathway is involved in ionizing radiation induction of c-*jun*, c-*myc* and c-*Ha-ras*, but not c-*fos*. The PKC pathway is also involved in induction of all four genes but only weakly for C-*Ha-ras*.

When PMA induction and ionizing radiation induction of the four IEGs are compared (Table 1), the set of pathways involved in the induction of c-*fos*, c-*jun*, and c-*Ha-ras* expression are qualitatively different; they are however, qualitatively similar for c-*myc* (Table 1). The PTK pathway appears to be a common pathway for the induction of all four immediate early genes by both PMA and ionizing radiation. Induction of c-*fos* by ionizing radiation differs from PMA in that it involves the calcium channel and does not involve ROI. For both transcripts of c-*jun*, induction by ionizing radiation differs from PMA in that it involves the PKC pathway. For c-*Ha-ras*, induction by ionizing radiation differs from PMA in that calcium channel, ROI pathway, and PKC pathway (weakly) are involved.

DISCUSSION

Activation of c-fos, c-jun, c-myc and c-Ha-ras by Low-Dose Ionizing Radiation:

We have previously reported that low dose ionizing radiation (0.1-2.0 Gy), where cell viability remains high, transiently activates transcription factor NF-κB in human lymphoblastoid 244 B cells; the DNA binding activity was maximum

after 0.5 Gy exposure at 8 h [21,22]. In this study, we have demonstrated the same levels of low dose ionizing radiation (0.25-2.0 Gy) differentially induce the expression of four protooncogenes, including c-*fos*, c-*jun*, c-*myc* and c-*Ha-ras*, in 244B cells, and that the induction was transient, reaching a maximum at 1 h and declining to the constitutive level within 4 h after exposure. The time course of the initial induction after ionizing radiation exposure was similar to that occurring after PMA treatment at a final concentration of 50 ng/ml. The low dose ionizing radiation and PMA inductions did not follow an exactly similar pattern of signal transduction pathway, however, for the four individual protooncogenes studied; of the four major pathways examined, the only one commonly utilized was the protein tyrosine kinase (PTK) dependent pathway. This study proves that the cellular response to any single etiological agent, including ionizing radiation, involves multiple gene activation and multiple and different signaling pathways.

a) Activation of Protooncogenes by Ionizing Radiation, and its Dose-dependence:

Recently, ionizing radiation exposures at high doses (5-50 Gy) were reported to induce (several-fold) the expression of protooncogenes of the c-*fos* and c-*jun* family, with a maximum expression after 50 Gy exposures in human HL-60 promyelocytic leukemia cells, U-937 monocytic leukemia cells and normal human AG-1522 diploid fibroblasts [13]; this dose is 100 times greater than studied here in 244B cells. Another study also showed c-*jun* induction in a human sarcoma cell line after 10 Gy exposure [23], a dose 20 times greater than studied here. In all of those studies, the high doses would have resulted in extensive DNA damage through direct and/or indirect action and extensive cell killing [24]. In contrast, after low dose ionizing radiation exposure of 244B human lymphoblastoid cells, where cell viability remains very high, we have demonstrated maximum expressions of c-*fos* after a 0.25 Gy exposure, and of c-*jun*, c-*myc* and c-*Ha-ras* after 0.5 Gy exposures. The induction of c-*fos* and c-*jun* then declined with increasing dose, being at near constitutive level after a 2.0 Gy exposure. The inductions of c-*myc* and c-*Ha-ras* both showed a biphasic expression, with initial maximums at 0.5 Gy, decreasing to near constitutive level at 1 Gy, and then

responding with a second high induction level (although not to the same level as after 0.5 Gy) after a 2.0 Gy exposure. The induction of protooncogenes after the low doses of ionizing radiation observed here is therefore much more relevant to the study of the effects of ionizing radiation on function, cell division and (if it were to occur in other systems) on cell differentiation. The induction observed after low dose ionizing radiation exposure could be part of a complex response by cells to protect themselves against oxidative and other injuries playing a yet to be defined role in cellular repair mechanisms, or it could be incidentally caused by activation of these repair mechanisms. It remains to be determined if different signalling pathways and mechanisms are involved at different ionizing radiation exposure levels. It is also possible that the induction is not related to the repair mechanism within a cell, but rather a means to accomplish intercellular communication to indicate that intracellular damage is occurring or has occurred.

It remains possible that the induction of protooncogenes after different doses of ionizing radiation is cell type specific, as well as dose dependent. This is suggested by the reports of an increased activation of the protooncogene c-*fos* after a very high dose (50 Gy) in HL-60, U-937 and normal human AG-1522 diploid fibroblast cells [13], but not after a 10 Gy exposure in a sarcoma cell line [23].

An additional finding of this study was that after low dose ionizing radiation (0.25-20 Gy) exposure or with PMA treatment, both transcripts of c-*jun* were induced in 244B cells. The low molecular weight (3.2 kb) c-*jun* transcript was equally activated at all low doses of ionizing radiation exposure or at all concentrations of PMA used in this study. Similar induction of both transcript of c-*jun* was shown after higher doses (5-50 Gy) in normal human AG-1522 cells [13]. The appearance of this low molecular weight (3.2 kb) c-*jun* transcript, however, was not apparent after higher doses (5-50 Gy) in both HL-60 promyelocytic leukemia cells and U-937 monocytic leukemia cells [13].

b) Time Course of Activation of Protooncogenes by Ionizing Radiation:

We have also demonstrated that the expression of protooncogenes after low dose ionizing radiation was transient, with a maximum consistently occurring at 1 h in this low dose range, followed (also) consistently by a considerable decline at 2 h and reaching to near constitutive levels at 4 h after exposure. In contrast, after higher doses studied in other laboratories, the expression of protooncogenes were shown to occur much later, and to be more persistent than seen after low dose exposure. For example after a 50 Gy exposure, the maximum inductions of c-*fos* and c-*jun* were shown to occur at 3 h after exposure, declining to a constitutive level only after 24 h [13]. These differences in the time course of induction after low-dose-range exposures and high-dose-range exposures leave open several possibilities, not the least of which is that the response observed after high doses is associated with cell death, and not meaningful to the outcomes in cells which continue to live (and which can go on to proliferate, differentiate, be repaired and mutated, or be transformed). Woloschak et al. [25] have demonstrated increased levels of PKC-specific mRNA 1h after exposure of SHE fibroblasts (at a dose even lower than reported here, 1 cGy), to x-rays or γ-rays (low-linear energy transfer) compared to fission spectrum neutrons (high LET) exposures.

Signal Transduction Pathways Involved in the Induction of Protooncogenes by Ionizing Radiation:

Another important aspect of this study was the investigation of the signaling pathways involved in the radiation induction of the four proto-oncogenes. This was performed 1 h after exposure to 0.5 Gy, and the pathways involved were compared to those involved after PMA treatment. The results showed that only the tyrosine kinase dependent signaling pathway is involved in the induction of all four protooncogenes, whether the cells are treated either with PMA or 0.5 Gy of ionizing radiation. Tyrosine phosphorylation is one of the early responses in the cell to a variety of stimuli, and was shown to be essential for cell activation in B lymphocytes [26]. Uckun et al. [27] have recently shown that tyrosine phosphorylation is a mandatory proximal step in the

radiation induced activation of protein kinase C signaling pathway, after higher doses, and in the activation of transcription factor NF-κB in human B lymphocyte precursors. This is therefore likely to occur in our 244B cells, where at least 50% of the NF-κB activation is associated with the ROI pathway. In addition, our results also show that (at least) three other pathways, the calcium channel-, ROIs-, and protein kinase C-pathways, are also involved in the ionizing radiation induction of protooncogenes at 0.5 Gy, but that the involvements are different for each protooncogene. For comparison, exposure of human sarcoma cells to 10 Gy resulted in c-*jun* induction which was shown to involve only the PKC pathway, and not calcium, serum or cAMP dependent protein kinases [23]. Thus, these protooncogenes are regulated by different specific interacting signaling pathways in response to different stimuli, with possible differences due to either dose and cell type.

The protooncogenes function as third messenger molecules in coupling short-term signals elicited by extracellular stimuli to long term adaptive changes in cell phenotype by regulating the expression of specific target genes [28]. Thus after low doses of radiation, where the cell survival is still high and the cells are capable of continuing to perform normal proliferative functions, the gene products (of stress responsive genes) might be interacting at the cell membrane level [8], triggering the membrane bound specific second messenger signaling cascades [29,30], and thereby participating in activation of the cell's DNA repair mechanism. Simultaneously and/or alternatively, the gene induction could directly or indirectly result in cell-to-cell communication by triggering the synthesis or release of various cytokines and other growth factors. For example, the proto- oncogene *ras* was reported to be involved in signal transduction pathways for interleukins IL-2, IL-3 and GMC-SF [31]. Induction of protooncogenes by various stimuli has also been shown to play a key role in the signal transduction cascade that regulates cell growth and differentiation. Several studies have demonstrated the association of the c-*myc* protooncogene with cell proliferation [32], apoptosis [33,34] and neoplasia [35,36]. Similarly,

c-*fos* and c-*jun* inductions are involved in various second messenger signalling pathways [37,38].

The data presented here strongly suggest that the activation of immediate early genes are part of the mammalian cell phenotypic response to ionizing radiation injury. The observation in viable cells is important because it has been hypothesized that modulation of the expression of protooncogenes can result in abnormal signal transduction, leading to transformation or oncogenesis.

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TABLE 1: EFFECT OF SECOND MESSENGER INHIBITORS ON PMA OR IONIZING RADIATION INDUCED IMMEDIATE EARLY GENES IN 244B HUMAN LYMPHOBLASTOID CELLS

Second Messenger Inhibitors	PMA (20 ng/ml)				Irradiation (0.5 Gy)			
	c-fos	*c-jun*	*c-myc*	*c-Ha-ras*	*c-fos*	*c-jun*	*c-myc*	*c-Ha-ras*
Nifedipine (Calcium Channel Blocker	-	+/+	+	-	+	+/+	+	+
NAC (reactive oxygen intermediate scavenger)	+	+/+	+	-	-	+/+	+	+
Calphostin C (protein kinase C inhibitor)	+	-/-	+	-	+	+/+	+	-
Genistein (protein tyrosine kinase inhibitor)	+	+/+	+	+	+	+/+	+	+

The percent inhibitorty effect was determined by comparing the respective transcript levels with that of PMA or 0.5 Gy alone induced mRNA levels

(+) = inhibition

(-) = no inhibition

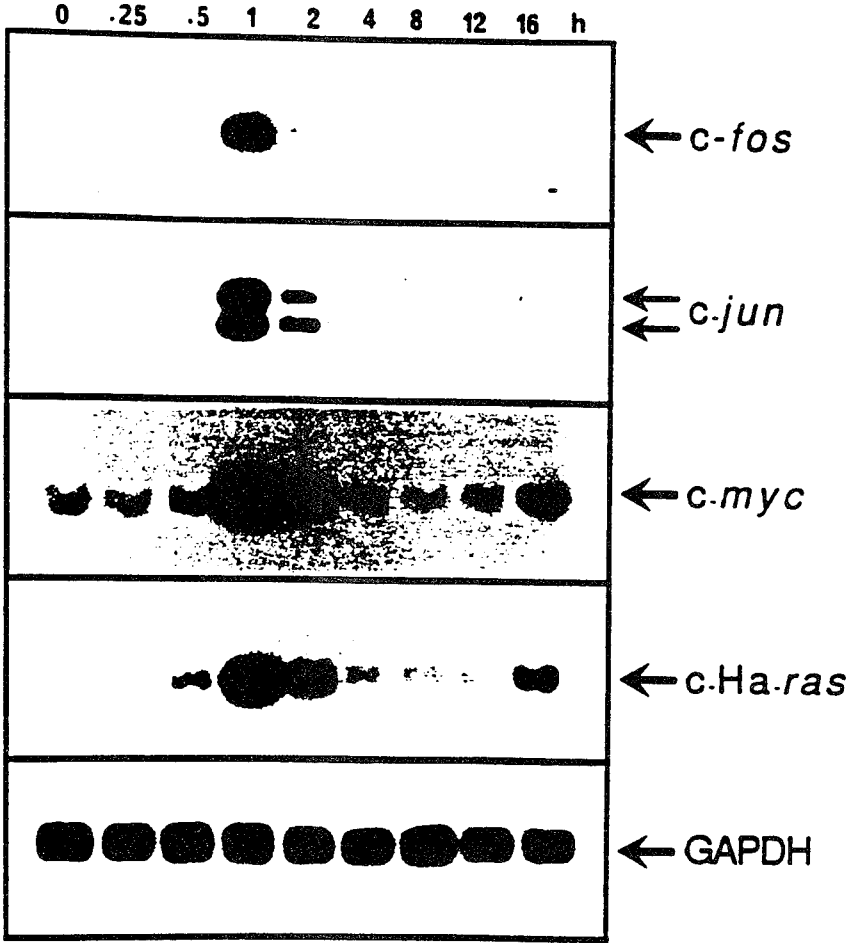

Figure 1: Kinetics of changes in c-*fos*, c-*jun*, c-*myc* and c-*Ha-ras* mRNA expression in 244B human lymphoblastoid cells after 0.5 Gy exposure. Northern blot analysis was carried out as described in the **Materials and Methods**. Autoradiogram of the filter hybridized to respective probes (c-*fos*, c-*jun*, c-*myc* and c-*Ha-ras*) is shown. Lowermost panel shows the autoradiogram of the same filter rehybridized with a probe specific for GAPDH.

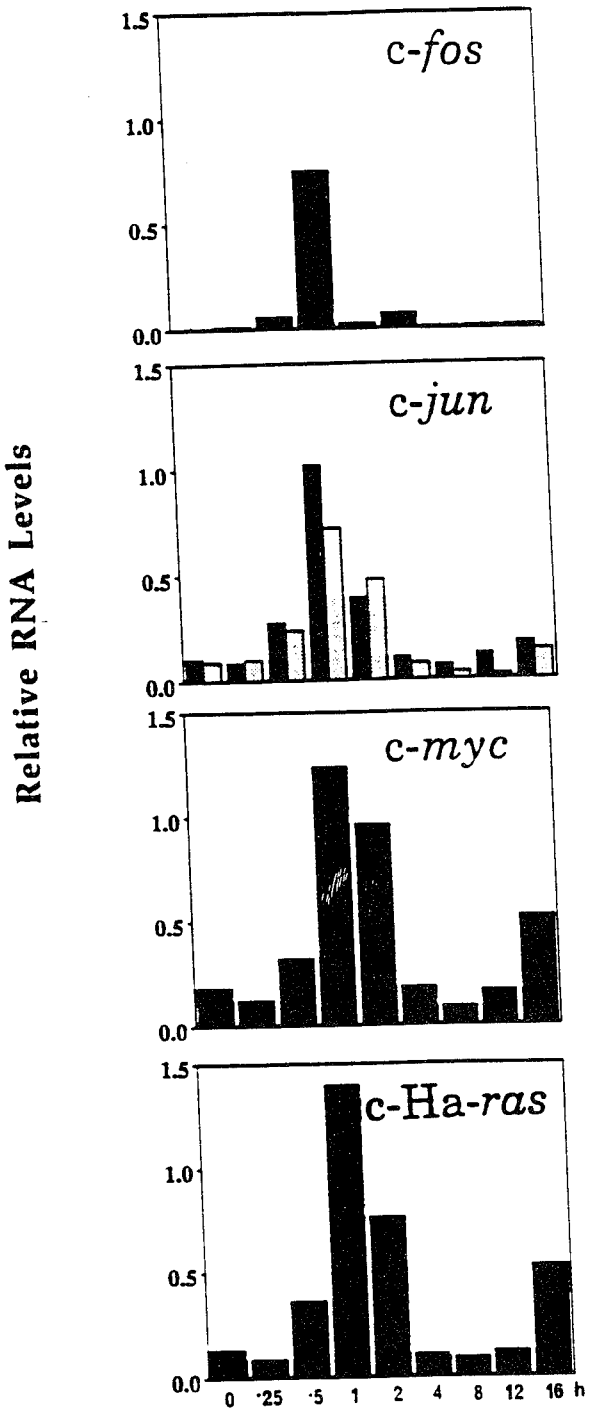

Figure 2: Densitometric analysis. Quantification of mRNA levels by densitometric analysis of the autoradiogram shown in **Figure 1**. Relative mRNA levels are the ratios at each time point of the mRNA level of the protooncogene to its respective housekeeping gene (GAPDH level).

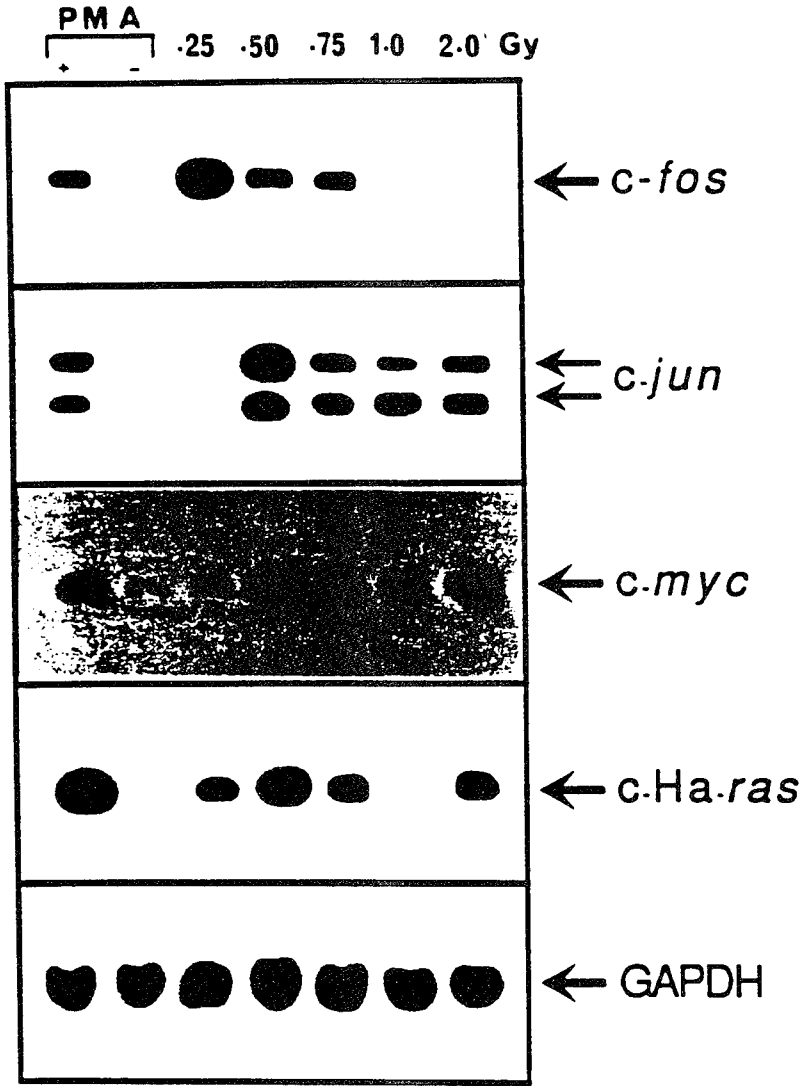

Figure 3: Analysis of c-*fos*, c-*jun*, c-*myc* and c-Ha-*ras* mRNA induction in 244B human lymphoblastoid cells. RNA was prepared from cells 1 h after irradiation (0.25, 0.5, 0.75, 1.0 and 2.0 Gy) or initiation of treatment with PMA (20 ng/ml). Northern blot analysis was carried out as described in **Materials and Methods.** The autoradiogram of the filter hybridized to the respective probes (c-*fos*, c-*jun*, c-*myc* and c-*Ha-ras*) is shown. The lowermost panel shows the autoradiogram of the same filter rehybridized with a probe specific for GAPDH.

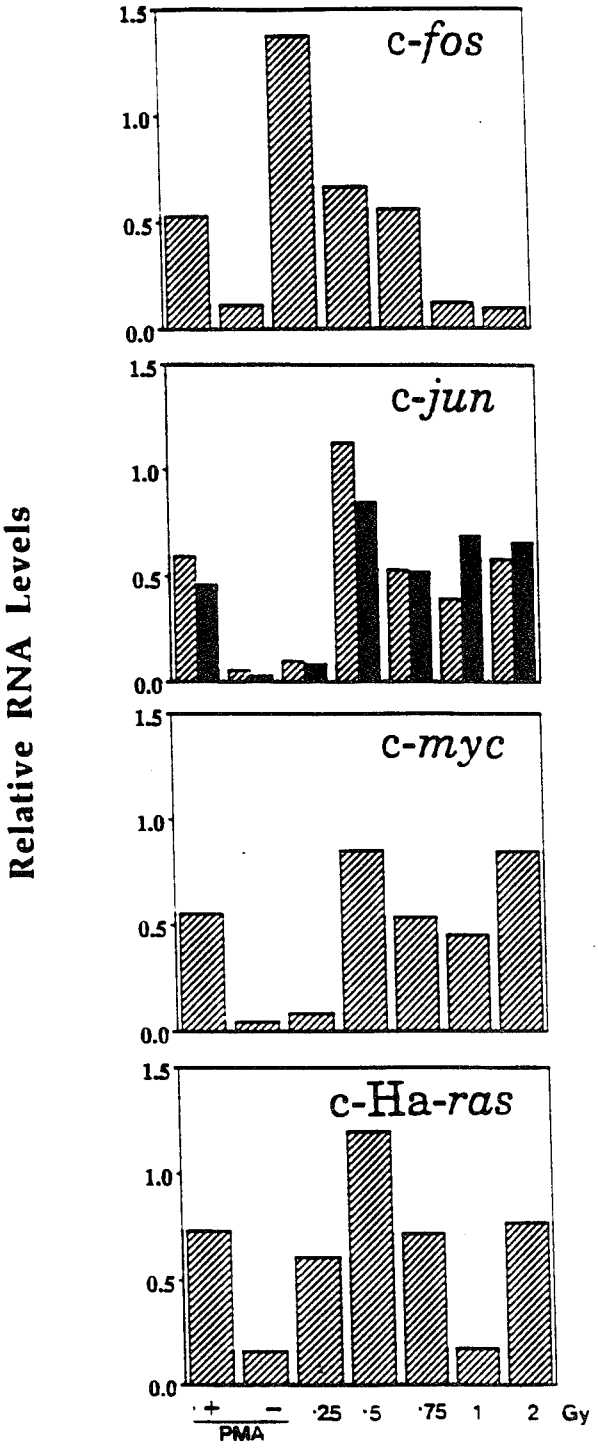

Figure 4: Densitometric analysis. Quantification of mRNA levels by densitometric analysis of the autoradiogram shown in **Figure 3**. Relative mRNA levels are the ratios at each dose of the mRNA level of the protooncogene to its respective housekeeping gene (GAPDH level).

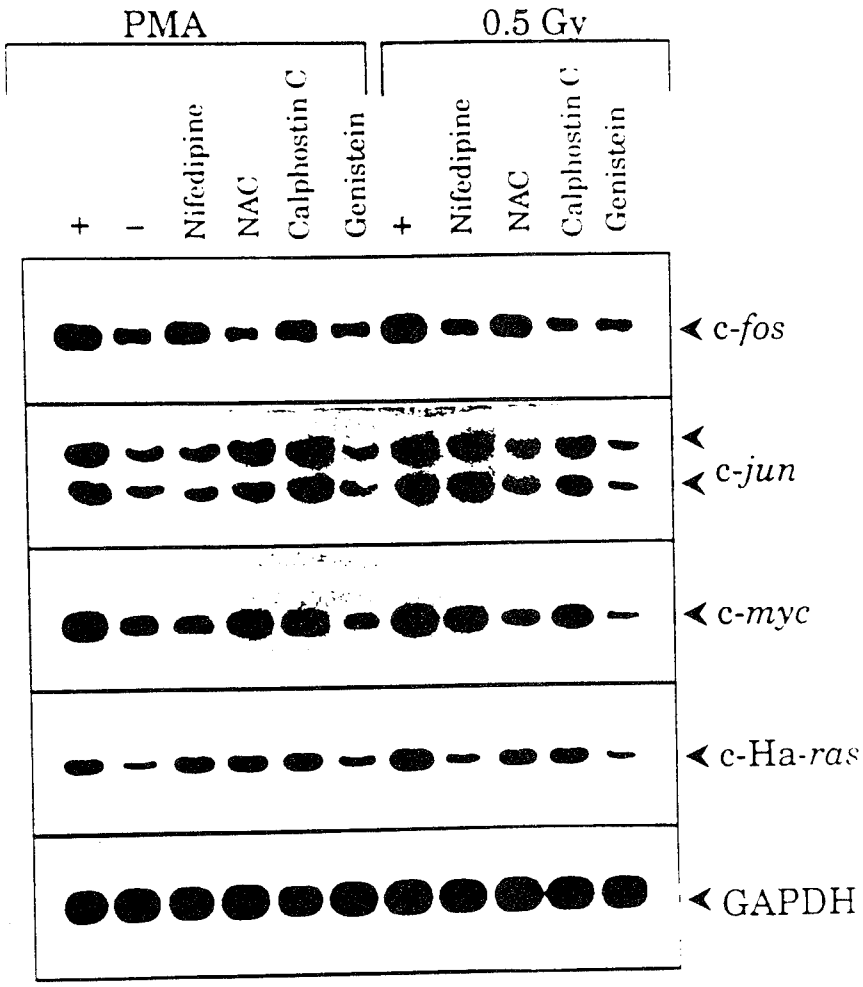

Figure 5: Analysis of c-*fos*, c-*jun*, c-*myc* and c-Ha-*ras* mRNA induction in 244B human lymphoblastoid cells in the presence or absence of various second messenger inhibitors. The 244B cells were treated with inhibitors of calcium dependent kinases (nifidipine, 50 μM) reactive oxygen intermediates (NAC, 30 mM), protein kinase C (calphostin, 50 nM) and protein tyrosine kinase (genestein 2.6 μM) for 30 min followed either by treatment with PMA (20 ng/ml) or exposure to 0.5 Gy. Total RNA was prepared from the cells collected 1 h after addition of PMA or radiation exposure and Northern blot analysis was performed as described in **Materials and Methods**. The autoradiogram of the filter hybridized to the respective probes (c-*fos*, c-*jun*, c-*myc* and c-Ha-*ras*) is shown. The lowermost panel shows the autoradiogram of the same filter rehybridized with a probe specific for GAPDH. Lanes 1 and 2 represent the induction of mRNA levels in the presence (+) or absence (-) of PMA only without addition of the inhibitors. Lane 7 represents the induction of mRNA levels in the presence (+) of 0.5 Gy only without inhibitors.

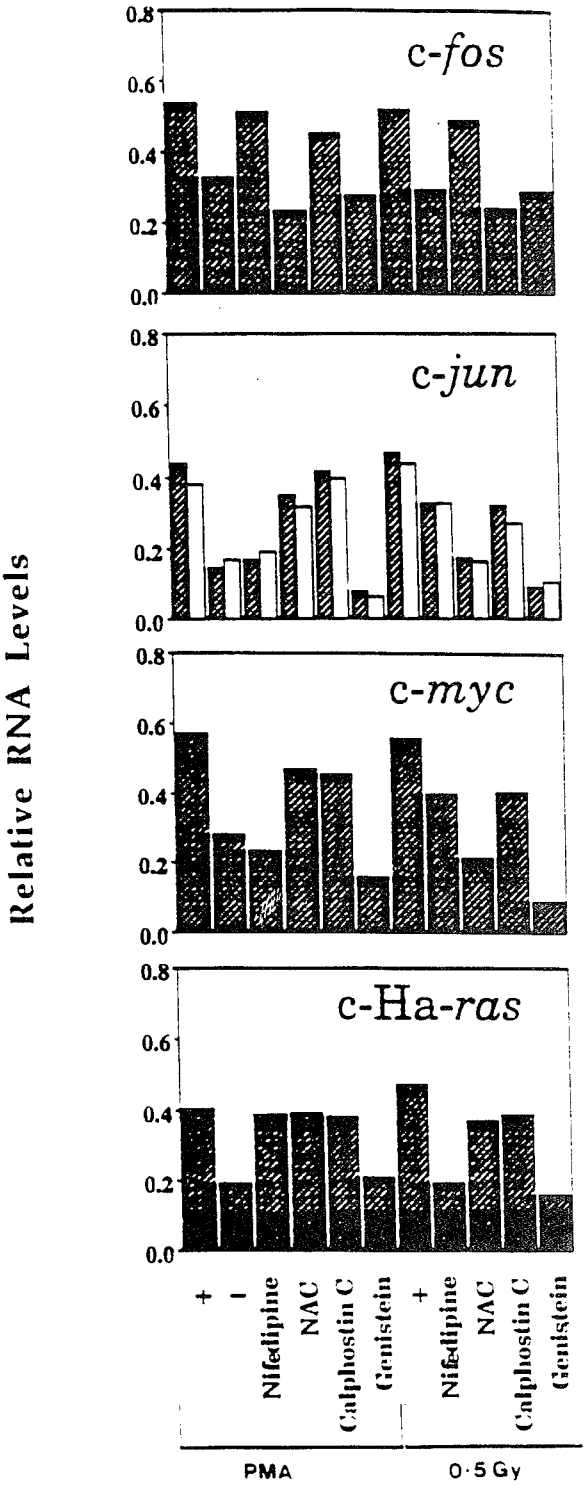

Figure 6: Densitometric analysis. Quantification of mRNA levels by densitometric analysis of the autoradiogram shown in **Figure 1**. Relative mRNA levels are the ratio for each inhibitor studied of the mRNA level of the protooncogene to its respective housekeeping gene (GAPDH level)

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Mutation Research 346 (1995) 23–31

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Melatonin protects human blood lymphocytes from radiation-induced chromosome damage

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Abstract

Cells in human peripheral blood were treated in vitro with increasing concentrations of melatonin (0.5 or 1.0 or 2.0 mM) for 20 min at 37 ± 1°C and then exposed to 150 cGy γ-radiation from a ^{137}Cs source. The lymphocytes which were pre-treated with melatonin exhibited a significant and concentration-dependent decrease in the frequency of radiation-induced chromosome damage as compared with the irradiated cells which did not receive the pre-treatment. The extent of the reduction in radiation-induced chromosome damage observed with 2.0 mM melatonin was similar to that found in lymphocytes pre-treated with 1.0 M dimethyl sulfoxide, a known free radical scavenger. Melatonin at 2.0 mM (a 500 × lower concentration) was as effective in decreasing the radiation-induced chromosome damage as dimethyl sulfoxide at 1.0 M. These observations may have implications for human protection against damage due to endogenously produced free radicals and also due to exposure to free radical producing physical and chemical mutagens and carcinogens.

Keywords: Melatonin; Gamma radiation; Free radical; Chromosome damage

1. Introduction

Melatonin, *N*-acetyl-5-methoxytryptamine (Fig. 1), is an evolutionarily highly conserved molecule, existing in organisms as diverse as algae and humans. In man, it is synthesized mainly by the pineal gland in the brain where it exhibits a circadian rhythm with maximum production occurring during the night (Ebadi, 1984; Reiter, 1991a). It participates in the regulation of a number of important physiological and pathological processes in mammals, and it reportedly has anti-aging and life-prolonging effects (Reiter, 1980, 1991b, 1992; Pierpoali et al., 1991). Of

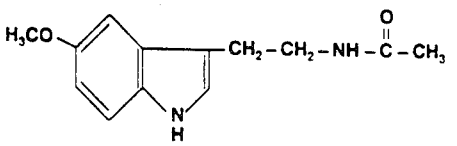

Fig. 1. Structure of the pineal hormone, melatonin.

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particular interest is the demonstration that melatonin strongly scavenges hydroxyl radicals ($^{\cdot}OH$) generated in vitro when hydrogen peroxide is exposed to ultraviolet light (Tan et al., 1993a).

It has long been known that the damaging effects of ionizing radiation on cellular DNA are brought about by both direct and indirect mechanisms. Direct action produces disruption of chemical bonds in the molecular structure of DNA while indirect effects result from highly reactive free radicals such as $^{\cdot}OH$, $^{\cdot}H$ and e^{-}_{aq} produced during the radiolysis of water, and their subsequent interaction with cellular DNA. Using radical scavenging compounds, several investigators have demonstrated that a significant proportion of radiation-induced biological effects are attributable to the indirect action, and that OH radicals are primarily responsible for these radiation effects (Roots and Okada, 1972; Okada et al., 1983; Littlefield et al., 1988). In this context, the radical scavenging ability of melatonin (Tan et al., 1993a) deserves further investigation. There is a continued requirement for the identification and development of effective, non-toxic, radical scavengers which can protect humans against radiation and other types of free radical genetic damage. Potential applications for these can be found, for example, in the protection of astronauts during solar flares, and in radiation oncology to protect normal tissues.

The present study is designed to test the hypothesis that pre-treatment of human blood lymphocytes in vitro with melatonin decreases the incidence of γ-radiation-induced chromosome damage.

2. Materials and methods

Six separate experiments were conducted at different times, each using a blood sample from a different donor.

The chemicals and tissue culture media used in these experiments included absolute ethanol, acetic acid, bromodeoxyuridine (BrdUrd), colcemid, dimethyl sulfoxide (DMSO), glutamine, melatonin (Regis Chemical Co., Morton Grove, IL), methanol, penicillin and streptomycin (Mediatech, Washington, DC), phytohemagglutinin (PHA) (Gibco), potassium chloride (KCl), RPMI 1640 medium (Mediatech) and fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS).

Experiments were conducted in a laboratory fitted with safe (yellow) lights to prevent the exposure of the chemicals and the cells to fluorescent light. The blood donors, three males and three females, were healthy and non-smoking volunteers aged between 30 and 50 years. Peripheral blood was collected in sterile vacutainer tubes containing heparin as an anticoagulant. From each sample, separate cultures were set up in T-25 sterile tissue culture flasks by mixing 0.8 ml whole blood with 9.2 ml pre-warmed RPMI 1640 medium containing 15% FBS, 5 U/ml penicillin, 5 μg/ml streptomycin, and 2 mM glutamine. The flasks were kept at 37 ± 1°C in a humidified atmosphere of 5% CO_2 and 95% air for approximately 20 min to equilibrate the cells at the incubator temperature. During this time, a fresh stock solution of melatonin was prepared in filter sterilized absolute ethanol and filter sterilized again (0.2 μM Nalgene syringe filter). Further dilutions were made in sterilized absolute ethanol. Equal volumes of the freshly prepared melatonin solutions were added to the cultures after the 20 min incubation, to give final concentrations of 0.5, 1.0 and 2.0 mM. As a positive control, DMSO, a known radical scavenger, was used at a final concentration of 1.0 M (Littlefield et al., 1988). The solvent control cultures received the same volume of absolute ethanol (final concentration 1%) as used in melatonin treated cultures. Also studied were controls which were unirradiated and untreated (see Table 1). All treatments were carried out at 37 ± 1°C in a humidified atmosphere of 5% CO_2 and 95% air for 20 min. The cells were then exposed to γ-radiation at room temperature using a ^{137}Cs GammaCell-40 Irradiator (Atomic Energy of Canada Ltd.). They were irradiated with a total dose of 150 cGy, delivered at a dose rate of 112.5 cGy/min. Immediately after irradiation, all cell suspensions were transferred to separate 50 ml sterile conical centrifuge tubes, diluted with 30 ml pre-warmed RPMI 1640 culture medium (prepared as above), centrifuged, and the supernatant aspirated.

Table 1
The effect of pre-treatment with melatonin on the frequency of chromosome aberrations induced by 150 cGy γ-radiation in human blood lymphocytes

Culture treatment	Abnormal cells					Exchange aberrations					Acentric fragments [a]				
	Observed	Expected	Difference	% decrease		Observed	Expected	Difference	% decrease		Observed	Expected	Difference	% decrease	
Blood donor C.F. (200 cells / culture)															
Mel 0.5 mM + 150 cGy	38	51	13	25.5	NS	31	47	16	34.0	*	13	22	9	40.9	NS
Mel 1.0 mM + 150 cGy	35	51	16	31.4	*	25	47	22	46.8		**	16	22	627.3	NS
Mel 2.0 mM + 150 cGy	18	52	34	65.4	***	16	47	31	66.0	***	4	23	19	82.6	***
DMSO 1.0 M + 150 cGy	20	51	31	60.8	***	13	47	34	72.3	***	10	22	12	54.5	**
Ethanol 1% + 150 cGy	54	52	−2	−3.8	NS	51	47	−4	−8.5	NS	15	23	8	34.8	NS
Blood donor E.K. (200 cells / culture)															
Mel 0.5 mM + 150 cGy	41	57	16	28.1	*	34	48	14	29.2	*	16	30	14	46.7	*
Mel 1.0 mM + 150 cGy	29	56	27	48.2	***	22	48	26	54.2		***	13	29	1655.2	***
Mel 2.0 mM + 150 cGy	25	57	32	56.1	***	16	48	32	66.7		***	11	30	1963.3	***
DMSO 1.0 M + 150 cGy	18	56	38	67.9	***	13	48	35	72.9		***	8	29	2172.4	***
Ethanol 1% + 150 cGy	51	57	6	10.5	NS	40	48	8	16.7	NS	30	30	0	0.0	NS
Blood donor M.M. (200 cells / culture)															
Mel 0.5 mM + 150 cGy	40	67	27	40.3	***	29	51	22	43.1		**	21	37	1643.2	*
Mel 1.0 mM + 150 cGy	33	67	34	50.7	***	21	51	30	58.8		***	15	37	2259.5	***
Mel 2.0 mM + 150 cGy	28	67	39	58.2	***	15	51	36	70.6		***	13	37	2464.9	***
DMSO 1.0 M + 150 cGy	18	68	50	73.5	***	14	51	37	72.5		***	9	38	2976.3	***
Ethanol 1% + 150 cGy	65	68	3	4.4	NS	53	51	−2	−3.9	NS	35	38	3	7.9	NS
Blood donor M.N. (200 cells / culture)															
Mel 0.5 mM + 150 cGy	44	61	17	27.9	*	34	49	15	30.6	*	18	24	6	25.0	NS
Mel 1.0 mM + 150 cGy	28	60	32	53.3	***	20	49	29	59.2		***	14	23	939.1	NS
Mel 2.0 mM + 150 cGy	25	60	35	58.3	***	16	49	33	67.3		***	11	23	1252.2	*
DMSO 1.0 M + 150 cGy	23	61	38	62.3	***	20	49	29	59.2		***	9	24	1562.5	**
Ethanol 1% + 150 cGy	57	61	4	6.6	NS	46	49	3	6.1	NS	20	24	4	16.7	NS
Blood donor P.R. (200 cells / culture)															
Mel 0.5 mM + 150 cGy	42	61	19	31.1	*	35	48	13	27.1	NS	12	27	15	55.6	**
Mel 1.0 mM + 150 cGy	31	61	30	49.2	***	23	48	25	52.1		***	12	27	1555.6	**
Mel 2.0 mM + 150 cGy	23	61	38	62.3	***	15	48	33	68.8		***	8	27	1970.4	***
DMSO 1.0 M + 150 cGy	19	62	43	69.4	***	13	48	35	72.9		***	7	28	2175.0	***
Ethanol 1% + 150 cGy	56	61	5	8.2	NS	46	48	2	4.2	NS	25	27	2	7.4	NS
Blood donor V.J. (200 cells / culture)															
Mel 0.5 mM + 150 cGy	45	67	22	32.8	**	34	57	23	40.4		**	18	30	1240.0	*
Mel 1.0 mM + 150 cGy	34	69	35	50.7	***	29	57	28	49.1		***	9	32	2371.9	***
Mel 2.0 mM + 150 cGy	20	68	48	70.6	***	18	57	39	68.4	***	8	31	23	74.2	***
DMSO 1.0 M + 150 cGy	15	67	52	77.6	***	10	57	47	82.5		***	9	30	2170.0	***
Ethanol 1% + 150 cGy	Not done					Not done					Not done				

Mel, Melatonin.
[a] Acentric fragments not associated with exchange aberrations.
Expected values are the sum of two individual treatments minus the control.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The cell pellet in each individual tube was then mixed with 9.2 ml complete RPMI 1640 medium supplemented with 15% FBS, 1% PHA, 5 U/ml penicillin, 5 μg/ml streptomycin, 2 mM glutamine, and 25 μM BrdUrd. The cell suspensions were transferred to separate T-25 culture flasks and incubated in the dark at 37 ± 1°C in a humidified atmosphere of 5% CO_2 and 95% air for 48 h. During the last 2 h of incubation, colcemid solution was added to all cultures at a final concentration of 0.1 μg/ml, to arrest the lymphocytes in mitosis.

Following the 48 h incubation, the cells were collected by centrifugation and resuspended in 10 ml KCl (75 mM) for 8 min; they were then fixed in three changes of 3:1 methanol:acetic acid mixture. Fixed cells were dropped onto clean microscopic slides, air dried and stained with the standard fluorescence-plus-Giemsa technique. At the completion of the experiments with blood samples from the six different donors, all slides were coded by an individual other than the scorer, and evaluated for chromosome damage. From each culture, 200 cells in their first mitotic division, as defined by the absence of harlequin staining, were examined.

Gaps and achromatic lesions less than the width of a chromatid were not included in the scoring. The numbers of abnormal cells showing chromosome damage, acentric fragments and exchange aberrations (dicentric, tricentric, ring, and tri/quadriradial chromosomes) were tabulated. Appropriate numbers of accompanying acentric fragments were assigned to each inter-chromosomal or inter-arm exchange chromosome; all excess acentric fragments were recorded separately.

The expected frequencies (abnormal cells showing chromosome damage, exchange aberrations and excess acentric fragments) in combined treatment groups, i.e., melatonin + 150 cGy, DMSO + 150 cGy and solvent control (ethanol) + 150 cGy, were computed as the sum of the effects of the two individual treatments minus the frequency in the control (unirradiated and untreated). The statistical significance of the de-

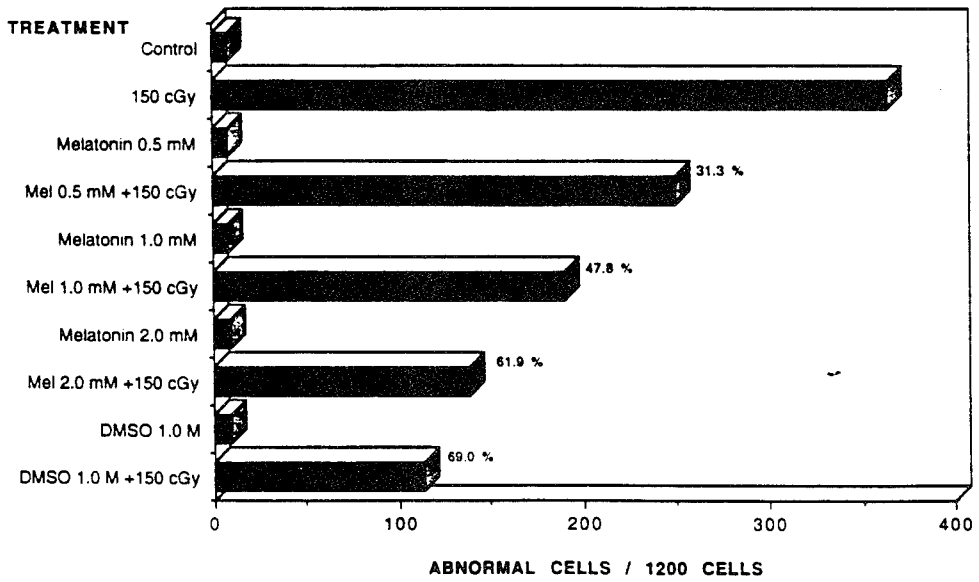

Fig. 2. Effect of pre-treatment with increasing doses of melatonin on radiation-induced abnormal cells (showing chromosome damage) in human peripheral blood lymphocytes. The percentages (listed at the top of each bar) are the extent of decreases based on the expected values, computed as the sum of the effects of the two individual treatments minus the control (unirradiated and untreated).

crease in the observed frequencies relative to the expected ones was evaluated using a one-tailed test (Vijayalaxmi and Burkart, 1989).

3. Results

The majority of the exchange aberrations were dicentric chromosomes. Seven tricentric chromosomes were recorded for the entire study. Few ring chromosomes were documented. Very few triradial and quadriradial chromosomes were observed. Polyploid cells were not found. The data collected upon the examination of 200 first division cells from each culture for the individual blood samples are presented in Table 1. The abnormal cells per 200 cells examined and the acentric fragments per 200 cells examined for individual agent treatments other than radiation never exceeded 2 (with one exception, = 3); the exchange aberrations without radiation was always zero. After different treatments, the overall response of the lymphocytes from the six donors was similar, although the absolute values were slightly different. The data from the six donors were pooled and are presented in Figs. 2–4.

Of the 1200 cells examined from the controls, 8 were abnormal cells showing chromosome damage (Table 1 and Fig. 2). Exposure to 150 cGy γ-radiation resulted in a significant increase in the incidence of abnormal cells: 364 in a total of 1200 cells ($p < 0.001$). Treatment of the unirradiated lymphocytes with 0.5, 1.0 and 2.0 mM melatonin, 1.0 M DMSO, or 1% ethanol had no significant effect on the numbers of abnormal cells. When the cells pre-treated with melatonin were exposed to radiation, a significant and concentration-dependent decrease in the frequency of abnormal cells was observed, as compared with the cells which received 150 cGy radiation alone: the average decreases were 31.3%, 47.8% and 61.9% in the lymphocytes pre-treated with 0.5, 1.0 and 2.0 mM melatonin, respectively. A similar reduction in the incidence of abnormal cells (average 69.0%) was observed when irradiated cells were pre-treated with 1.0 M DMSO. Ethanol (1%) has no significant effect on radiation-induced chromosome damage.

Exchange-type aberrations were not observed in a total of 1200 control cells or in a similar number of cells treated with either 0.5, 1.0 or 2.0 mM melatonin, or 1.0 M DMSO, or in 1000 cells

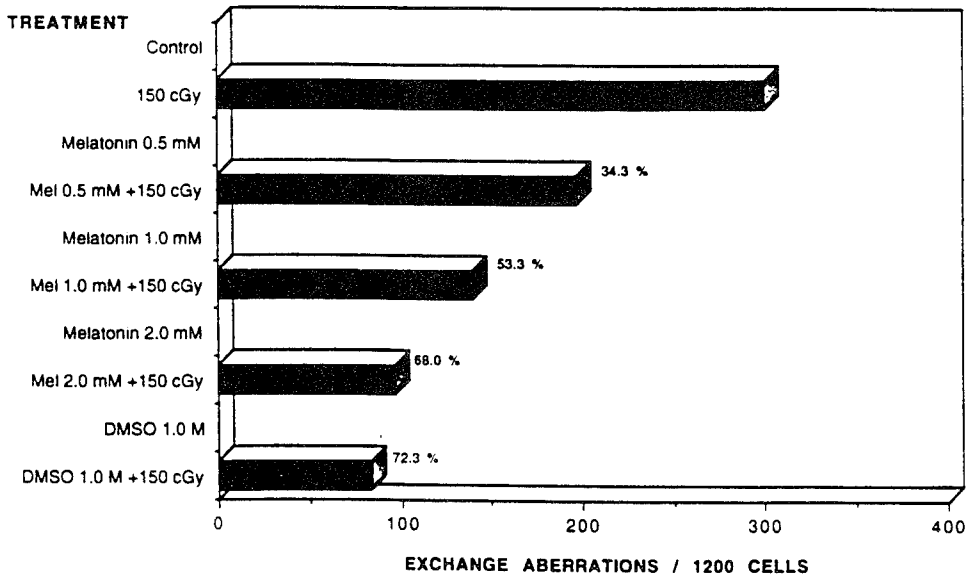

Fig. 3. Effect of pre-treatment with increasing doses of melatonin on radiation-induced exchange chromosomes in human peripheral blood lymphocytes. The percentages (listed at the top of each bar) are the extent of decreases based on the expected values, computed as the sum of the effects of the two individual treatments minus the control (unirradiated and untreated).

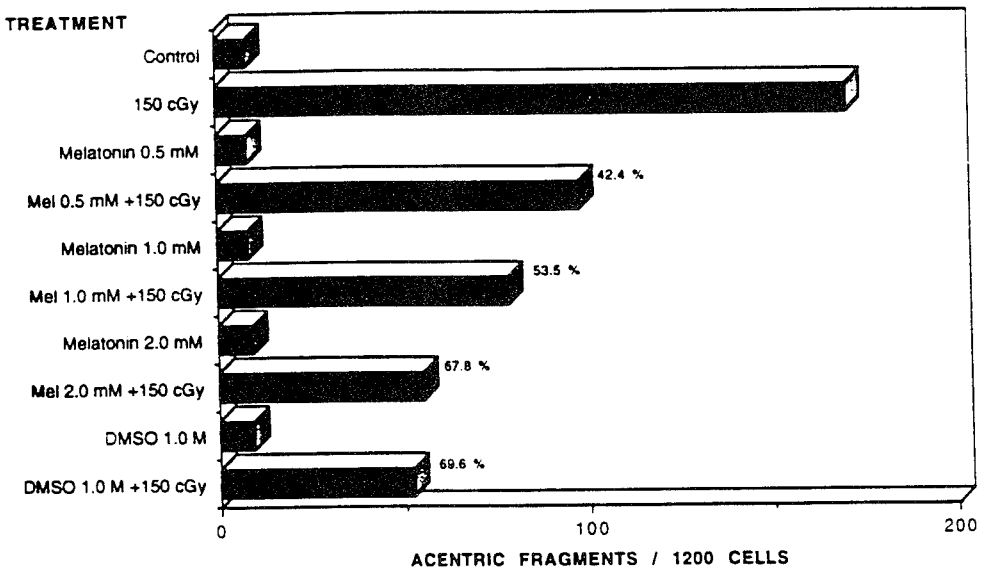

Fig. 4. Effect of pre-treatment with increasing doses of melatonin on radiation-induced acentric fragments in human peripheral blood lymphocytes. The percentages (listed at the top of each bar) are the extent of decreases based on the expected values, computed as the sum of the effects of the two individual treatments minus the control (unirradiated and untreated).

treated with 1% ethanol (Table 1, Fig. 3). Exposure of the lymphocytes to 150 cGy γ-radiation resulted in a significant increase in the incidence of exchange chromosomes: 300 in a total of 1200 cells ($p < 0.001$). In contrast, a significant and concentration-dependent decrease in the frequency of exchange aberrations was observed when cells pre-treated with melatonin were exposed to 150 cGy radiation. The average decreases were 34.3%, 53.3% and 68.0% in the lymphocytes pre-treated with 0.5, 1.0 and 2.0 mM melatonin, respectively. A slightly greater reduction in the incidence of exchange aberrations (average 72.3%) was observed in the cells pre-treated with 1.0 M DMSO and then exposed to 150 cGy radiation. The numbers of radiation-induced exchange aberrations were not significantly different among the cells with and without ethanol (1%) pre-treatment in the two blood samples studied.

There were 8 acentric fragments in a total of 1200 control cells (Table 1 and Fig. 4). Exposure of the cells to 150 cGy γ-radiation resulted in a significant increase in the incidence of acentric fragments: 170 in a total of 1200 cells ($p < 0.001$). Treatment of the cells with 0.5, 1.0 and 2.0 mM melatonin or 1.0 M DMSO, or 1% ethanol had no significant effect on the numbers of acentric fragments. When the cells pre-treated with melatonin were exposed to radiation, a significant and concentration-dependent decrease in the frequency of acentric fragments was observed, as compared with the cells which received 150 cGy radiation alone: the average decreases were 42.4%, 53.5% and 67.8% in the lymphocytes pre-treated with 0.5, 1.0 and 2.0 mM melatonin, respectively. A similar reduction in the incidence of acentric fragments (average 69.6%) was observed when the cells pre-treated with 1.0 M DMSO were exposed to 150 cGy radiation. The numbers of radiation-induced acentric fragments were not significantly different among the cells with and without ethanol (1%) pre-treatment in the two blood samples studied.

4. Discussion

The present study is the first to demonstrate the ability of melatonin to significantly decrease γ-radiation-induced chromosome damage in human cells in vitro. Two hypotheses can be pro-

posed for the observed protective effect. First, melatonin may directly protect against chromosome damage by scavenging the free radicals generated by ionizing radiation (before they induce damage to the genetic material), i.e., the extent of primary damage in cellular DNA may be significantly reduced. Secondly, melatonin may have indirectly altered the final amount of chromosome damage by activating oxidative repair enzymes so that the damaged DNA is repaired more rapidly in irradiated cells pre-treated with melatonin. The genetic end-point used in the current investigation, i.e., chromosome damage, does not provide information either on the extent of primary DNA damage, or on the kinetics of DNA repair, since the primary DNA damage that is left unrepaired or misrepaired was identified as microscopically visible chromosome aberrations. Techniques are now available to investigate both of these hypotheses, i.e., to measure the extent of primary DNA damage and to determine the kinetics of DNA repair in individual human cells (Singh et al., 1990; Vijayalaxmi et al., 1992). Such studies, which are in progress in our laboratory, will give insight into the mechanism(s) of the protective action of melatonin on radiation-induced chromosome damage in human blood lymphocytes.

The published literature on melatonin indicates that either or both of the proposed hypotheses are valid. The small size and high lipophilicity of the melatonin molecule permits its diffusion through biomembranes easily (Reiter, 1991c). Studies on the distribution of melatonin in a number of tissues in several different species have shown that melatonin is localized in higher concentrations in the nucleus than in other subcellular compartments (Mennenga et al., 1990, 1991). In mammalian tissues, evidence from both immunocytochemical and radioimmunoassay studies indicates that endogenously produced or exogenously administered melatonin is more highly concentrated in the nucleus than in the cytosol (Menendez-Pelaez and Reiter, 1993). The tendency of melatonin to diffuse and accumulate within the nucleus along with its ability to scavenge free radicals (Tan et al., 1993a) would provide an effective and direct means of on-site protection for the lymphocytes against radiation-induced genetic damage. Melatonin also appears to activate at least one cellular enzymatic antioxidant defense mechanism, the induction of glutathione peroxidase (Janiaud, 1987): the activation of this enzyme reduces OH formation by metabolizing its precursor, H_2O_2. Other enzymes which are involved in DNA repair might also be induced by melatonin and such enzymes would facilitate rapid repair of the damaged DNA; currently, information on this aspect is lacking in the literature.

Three in vivo studies conducted with melatonin are worth mentioning here. Tan et al. (1993b) have treated Sprague-Dawley rats with safrole (300 mg/kg), an indirect acting carcinogen capable of inducing DNA damage, at least in part due to its ability to produce reactive oxygen species (Miller, 1983), with and without co-treatment with a low (0.2 mg/kg) or a high (0.4 mg/kg) dose of melatonin. When examined 24 h later, rats treated only with safrole exhibited extensive DNA damage in their liver as revealed by the quantity of DNA adducts (measured with the ^{32}P-postlabeling assay). Rats which were co-treated with a low dose of melatonin showed a 50% reduction in DNA adducts, while in those given the high dose of melatonin, the DNA adducts were hardly detectable. A dose-response relationship between serum melatonin levels and the ability of safrole to induce DNA adducts was observed. When the study was extended (Tan et al., 1994), even endogenous physiological levels of melatonin from the pineal gland significantly reduced DNA adduct formation in rats treated with safrole. The investigators related their observations to the radical scavenging capability of melatonin, and possibly also to its inhibitory effect on the mixed function oxidase, cytochrome P-450. More recently, Blickenstaff et al. (1994) reported an increased survival of male Swiss ND4 mice injected with melatonin (250 mg/kg) 30 min prior to irradiation with 950 cGy of 6 mV photons produced by a linear accelerator; nine of the 21 mice which were pre-treated with melatonin survived over a 30 day period while all 20 mice which were not pre-treated with melatonin died by day 12. These investigators also found that two ho-

mologs of melatonin were significantly more radioprotective than melatonin; 19 of the 20 mice treated with hexanoic amide, and 20 of the 21 mice injected with octanoic amide, survived over a 30 day period.

Our observations in human blood lymphocytes treated with melatonin in vitro, as well as the in vivo effects demonstrated by Tan et al. (1993b, 1994) in rats and Blickenstaff et al. (1994) in mice, are important with regard to free radical scavenging and radioprotection. In the present study, the response obtained with 2.0 mM melatonin was similar to that observed with 1.0 M DMSO, a known radical scavenger. The data indicate that melatonin was as effective, at a 500 × lower concentration, in decreasing the radiation-induced chromosome damage as 1.0 M DMSO. Since melatonin is not foreign to the human body, these results should provide impetus for further research leading to the use of melatonin, and the biological activity of this endogenously synthesized pineal hormone, in protection of the genome from natural and other DNA damaging agents.

Acknowledgements

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MARKED REDUCTION OF RADIATION-INDUCED MICRONUCLEI IN HUMAN BLOOD LYMPHOCYTES PRE-TREATED WITH MELATONIN

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ABSTRACT

Vijayalaxmi., R.J. Reiter, E. Sewerynek, B. Poeggeler, B.Z. Leal and M.L. Meltz. Marked reduction of radiation-induced micronuclei in human blood lymphocytes pre-treated with melatonin. *Radiat. Res.*

Human peripheral blood lymphocytes which were pre-treated in vitro with melatonin, an endogenously synthesized pineal hormone, for 20 minutes at 37±1°C exhibited a significant and concentration-dependent reduction in the frequency of gamma radiation-induced micronuclei as compared with irradiated cells which did not receive the pre-treatment. The extent of the reduction observed with 2.0 mM melatonin was similar to that found in lymphocytes pre-treated for 20 minutes with 1.0 M dimethylsulfoxide, a known free radical scavenger. These observations indicate that melatonin may have an active role in human protection against genetic damage due to endogenously produced free radicals, and also may be of use in reducing damage due to exposure to free radical-generating physical and chemical mutagens and carcinogens.

INTRODUCTION

Several investigators have demonstrated the ability of free radical scavenging compounds to protect cellular DNA against a significant proportion of the indirect effects of ionizing radiation, where hydroxyl radicals are believed to be one of the primary active species responsible (1-3). Recently, it was reported that melatonin, an endogenously synthesized pineal hormone (4,5), scavenges hydroxyl radicals generated *in vitro* by hydrogen peroxide exposed to ultraviolet light (6). Supporting evidence for the protective action of melatonin comes from *in vivo* studies in rodents. Melatonin inhibited the formation of DNA adducts in the liver of rats treated with safrole (7,8), an indirect acting chemical carcinogen which damages DNA, at least in part due to its ability to generate reactive oxygen species (9). In rats treated with a glutathione depleting drug, melatonin was shown to prevent cataract formation (10), a process attributed to free radical-induced damage to lenticular protein (11). Blickenstaff et al. (12) have demonstrated an increased survival of irradiated mice co-treated with melatonin. Recently, we have investigated the radioprotective effect of melatonin in human blood lymphocytes *in vitro* and observed that pre-treatment with melatonin for 20 minutes at $37\pm1^{\circ}C$ significantly reduced the incidence of chromosome aberrations resulting from a dose of 1.5 Gy ^{137}Cesium gamma radiation (13).

In recent years, the micronucleus technique, which quantitates micronuclei in cytokinesis blocked binucleate cells (14), has increasingly been used to assess the genotoxicity induced *in vitro* and/or *in vivo* in human blood lymphocytes (15-19). The technique has been used to measure and compare the genotoxicity induced in the blood lymphocytes of the same individuals after *in vitro* and *in vivo* exposure to ionizing radiation (20). Since the frequencies of micronuclei in control and irradiated human lymphocytes were substantially higher than the incidences of chromosome aberrations, probable differences in

the mechanisms involved in their formation has been suggested (21). Hence, this study was designed to investigate whether pre-treatment with melatonin, in vitro, offers protection against gamma radiation-induced micronuclei in cytokinesis blocked binucleate human blood lymphocytes, and for comparison with the incidences of chromosome aberrations measured earlier (13).

MATERIALS AND METHODS

Three separate experiments were conducted, each using a blood sample from a different non-smoking, healthy donor. From each sample, separate cultures were set up by mixing 0.8 ml whole blood with 9.2 ml pre-warmed RPMI 1640 medium (Mediatech, Washington DC) containing 15% fetal bovine serum (JRH Biosciences, Lenexa, KS), 5 U/ml penicillin, 5 µg/ml streptomycin, and 2 mM glutamine (Mediatech). Cells were treated with freshly prepared melatonin (Regis Chemical Co, Morton Grove, IL; dissolved in sterile absolute ethanol) to give final concentrations of 0.5, 1.0 and 2.0 mM, 1.0 M dimethylsulfoxide (DMSO) (ATCC, Rockville, MD), or ethanol solvent (1%), for 20 minutes at 37±1° C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were then exposed at room temperature to 1.5 Gy gamma radiation, delivered at a dose rate of 1.125 Gy/min (^{137}Cs GammaCell-40 Irradiator, Atomic Energy of Canada Ltd.).

Immediately after irradiation, all cells were washed and separate cultures re-incubated in the medium (described above) supplemented with 1% phytohemagglutinin (Gibco, Grand Island, NY) at 37±1° C in a humidified atmosphere of 5% CO_2 and 95% air. Cytochalasin B (Sigma, St. Louis, MO) was added to all cultures (4 µg/ml) at 44 hours to block the dividing cells in cytokinesis. Twenty eight hours after the addition of cytochalasin B, cells were collected, treated with 0.8% sodium citrate for 3 to 5 minutes and fixed in 5:1 methanol acetic acid mixture (treatments with 0.8% sodium citrate and 5:1 methanol:acetic acid resulted in cells with better preserved cytoplasm as compared with the

conventional use of 75 mM potassium chloride and 3:1 methanol:acetic acid). Fixed cells were dropped gently onto clean microscope slides, air dried and stained with 4% Giemsa (Sigma) using standard procedures. All slides were coded by an individual other than the scorer, and evaluated at 1000x magnification for the frequency of micronuclei in cytokinesis blocked binucleate cells with well-preserved cytoplasm.

Binucleate cells were selected on the basis of having two distinct nuclei of approximately equal size which may be attached by a fine nucleoplasmic bridge or overlapped slightly (touching each other at the edges). The micronuclei scored were located within the cytoplasm of a binucleate cell and were non refractile. They were morphologically identical to the main nuclei, but smaller, i.e., with a diameter between 1/16 to 1/4 of the main nuclei, and were not linked to the main nuclei via a nucleoplasmic bridge (22). From each culture, 2000 binucleate cells were examined to record the frequency of cells with one (C1MN), two (C2MN) or three (C3MN) micronuceli. The number of binucleate cells containing micronuclei in each treatment group was assessed as (C1MN)+(C2MN)+(C3MN). The total number of micronuclei observed in each treatment group were derived from (1xC1MN)+(2xC2MN)+(3xC3MN). The expected micronuclei frequencies in combined treatment groups, i.e., melatonin+1.5 Gy, DMSO+1.5 Gy, and ethanol+1.5 Gy, were computed as the sum of the effects of the two individual treatments minus the frequency in controls (unirradiated and untreated). Statistical significance in the observed frequencies relative to the expected incidences was evaluated using a one-tailed test (23).

RESULTS

For each treatment group, the number of binucleate cells containing micronuceli, the frequency distribution of binucleate cells with one, two and three micronuclei and the total number of micronuclei, in each of the three blood samples studied are

presented in Table 1. Data on the response of lymphocytes pre-treated with melatonin, DMSO and ethanol, to 1.5 Gy gamma irradiation, are given in Table 2.

In all cultures, the proportion of lymphocytes that were binucleate ranged from 40-55%. In the three blood samples studied, binucleate cells containing more than one micronucleus were not found in control cells (unirradiated and untreated) or in cells treated with 0.5, 1.0 or 2.0 mM melatonin, 1.0 M DMSO, or 1% ethanol. Exposure of the lymphocytes to 1.5 Gy gamma radiation resulted in a significant increase in the number of binucleate cells with one, two and three micronuclei ($p<0.001$). When the cells were pre-treated with melatonin prior to exposure with 1.5 Gy of gamma radiation, a significant and concentration-dependent reduction in the number of binucleate cells with micronuclei were observed; the average decreases were 32.5%, 45.4% and 61.6% in the lymphocytes pre-treated with 0.5, 1.0 and 2.0 mM melatonin, respectively (Table 2). An average decrease of 63.1% was also detected in irradiated cells which were pre-treated with 1.0 M DMSO; no such reduction was found in irradiated cells which were pre-treated with 1% ethanol (Table 2).

The total number of micronuclei in 2000 control cells or in 2000 cells treated with 0.5, 1.0 or 2.0 mM melatonin, 1.0 M DMSO, or 1% ethanol in the three blood samples studied ranged from 21 to 34: these frequencies are similar to those published in the literature for control cells and indicate no significant effect of the treatments with melatonin, DMSO or ethanol alone. Exposure of the lymphocytes to 1.5 Gy gamma radiation resulted in a significant increase in the total number of micronuclei in each of the three blood samples studied; the values ranged from 334 to 379 ($p<0.001$) (Table 1). In contrast, a significant and concentration-dependent reduction in the total number of micronuclei was detected when cells pre-treated with melatonin were exposed to 1.5 Gy gamma radiation; the average decreases were 36.0%, 50.0% and 63.7% in the lymphocytes pre-treated with 0.5,

1.0 and 2.0 mM melatonin, respectively (Table 2). An average decrease of 65.2% was also detected in irradiated cells which were pre-treated with 1.0 M DMSO; no such reduction was observed in irradiated cells which were pre-treated with 1% ethanol (Table 2).

DISCUSSION

The data presented here demonstrate the protective effect of melatonin against gamma radiation-induced micronuclei in human blood lymphocytes. This effect appears to be a direct one, since the treatment prior to exposure to gamma radiation lasted only for 20 minutes. Lopez-Gonzales et al. (24) reported that the rate at which melatonin binds to human blood lymphocytes was rapid at 37°C, reaching a maximal value in 5-10 minutes. High affinity-binding sites for melatonin have been reported in purified cell nuclei of rat liver (25,26). Menendez-Pelaez and Reiter (27) have demonstrated that both exogenously administered and endogenously synthesized melatonin were more highly concentrated in the nucleus than in the cytosol of mammalian tissues. These observations provide ample evidence for the rapid diffusion of melatonin into the nucleus, where it would be available to protect radiation-induced genetic damage. It has also been reported that melatonin activates glutathione peroxide, an enzyme which reduces hydroxyl radical formation by metabolizing its precursor, hydrogen peroxide (28); this phenomenon may or may not be involved in the rapid response observed here. It is also not known whether melatonin activates any of the enzymes which are directly involved in the repair of damaged DNA, another mechanism by which melatonin could reduce the number of micronuclei resulting from radiation exposure.

The average numbers of micronuclei observed in control and irradiated (1.5 Gy) cells in this study were 0.013/cell and 0.180/cell, respectively. The mean numbers of acentric fragments (not associated with exchange aberrations) in control and irradiated (1.5 Gy) cells obtained in our earlier study involving

the same three blood donors were 0.008/cell and 0.148/cell (13). In all other treatment groups, the incidence of micronuclei were found to be higher than the frequency of acentric fragments. These data, taken together, indicate that all of the acentric fragments might have been incorporated into the formation of micronuclei, and that the excess micronuceli may be caused by other mechanisms, such as the failure of whole chromosome(s) to segregate into daughter nuclei at the time of cell division. Immunofluorescence staining of the micronuclei using antikinetochore antibodies or fluorescence in situ hybridization of micronuclei with centromeric DNA probes (29-30) will help to differentiate the two mechanisms. Such techniques have been been applied to radiation studies (31).

The extent of reduction in radiation-induced micronuclei in the lymphocytes pre-treated with melatonin demonstrated in this study is very similar to the extent of decrease in the incidence of chromosome aberrations observed in our earlier investigation (13). Although the doses of melatonin used in these studies are several-fold higher than the pysiological levels of melatonin found in the human blood (5-18 pg/ml and 40-103 pg/ml during the daytime and night time, respectively) (32), the data obtained confirm the radioprotective effect of melatonin. Since melatonin is not foreign to the human body, its ability to protect the human genome from endogenous and exogenous free radical generating genotoxic agents deserves further investigation.

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TABLE 1: Frequency distribution of binucleate cells with one, two or three micronuclei (MN) in human blood lymphocytes pre-treated with melatonin and then exposed to 1.5 Gy gamma radiation.

Culture Treatment	Binucleate Cells with MN (*)	C1MN	C2MN	C3MN	Total MN (**)
Blood Donor M.M. (2000 Binucleate Cells/Culture) :					
Control	**21**	21	0	0	**21**
1.5 Gy	**324**	276	41	7	**379**
Melatonin 0.5 mM	**23**	23	0	0	**23**
Melatonin 1.0 mM	**25**	25	0	0	**25**
Melatonin 2.0 mM	**24**	24	0	0	**24**
DMSO 1.0 M	**25**	25	0	0	**25**
Ethanol 1%	**24**	24	0	0	**24**
Mel 0.5 mM +1.5 Gy	**221**	200	17	4	**246**
Mel 1.0 mM +1.5 Gy	**173**	165	8	0	**181**
Mel 2.0 mM +1.5 Gy	**121**	107	12	2	**137**
DMSO 1.0 M +1.5 Gy	**115**	98	16	1	**133**
Ethanol 1% +1.5 Gy	**298**	252	39	7	**351**
Blood Donor M.N. (2000 Binucleate Cells/Culture) :					
Control	**30**	30	0	0	**30**
1.5 Gy	**309**	264	35	10	**364**
Melatonin 0.5 mM	**31**	31	0	0	**31**
Melatonin 1.0 mM	**33**	33	0	0	**33**
Melatonin 2.0 mM	**31**	31	0	0	**31**
DMSO 1.0 M	**34**	34	0	0	**34**
Ethanol 1%	**33**	33	0	0	**33**
Mel 0.5 mM +1.5 Gy	**216**	204	11	1	**229**
Mel 1.0 mM +1.5 Gy	**176**	170	5	1	**183**
Mel 2.0 mM +1.5 Gy	**127**	120	6	1	**135**
DMSO 1.0 M +1.5 Gy	**122**	115	7	0	**129**
Ethanol 1% +1.5 Gy	**302**	262	31	9	**351**
Blood Donor P.R. (2000 Binucleate Cells/Culture) :					
Control	**26**	26	0	0	**26**
1.5 Gy	**306**	281	22	3	**334**
Melatonin 0.5 mM	**26**	26	0	0	**26**
Melatonin 1.0 mM	**29**	29	0	0	**29**
Melatonin 2.0 mM	**30**	30	0	0	**30**
DMSO 1.0 M	**26**	26	0	0	**26**
Ethanol 1%	**25**	25	0	0	**25**
Mel 0.5 mM +1.5 Gy	**199**	185	11	3	**216**
Mel 1.0 mM +1.5 Gy	**169**	160	7	2	**180**
Mel 2.0 mM +1.5 Gy	**116**	111	4	1	**122**
DMSO 1.0 M +1.5 Gy	**112**	108	4	0	**116**
Ethanol 1% +1.5 Gy	**299**	273	24	2	**327**

C1MN: Binucleate Cells with 1 Micronucleus

C2MN: Binucleate Cells with 2 Micronuclei

C3MN: Binucleate Cells with 3 Micronuclei

(*) = (C1MN) + (C2MN) + (C3MN)

(**) = (1xC1MN) + (2xC2MN) + (3xC3MN)

TABLE 2: The incidence of micronuclei in human blood lymphocytes pre-treated with melatonin and then exposed to 1.5 Gy gamma radiation.

Culture Treatment	Binucleate Cells with Micronuclei (*)					Total Micronuceli (**)				
	Obsserved	Expected	Difference (Exp-Obs)	% Decrease		Observed	Expected	Difference (Exp-Obs)	% Decrease	
Blood Donor M.M. (2000 Binucleate Cells/Culture) :										
Mel 0.5 mM +1.5 Gy	**221**	326	105	32.2	***	**246**	381	135	35.4	***
Mel 1.0 mM +1.5 Gy	**173**	328	155	47.3	***	**181**	383	202	52.7	***
Mel 2.0 mM +1.5 Gy	**121**	327	206	63.0	***	**137**	382	245	64.1	***
DMSO 1.0 M +1.5 Gy	**115**	328	213	64.9	***	**133**	383	250	65.3	***
Ethanol 1% +1.5 Gy	**298**	327	29	8.9	*NS*	**351**	382	31	8.1	*NS*
Blood Donor M.N. (2000 Binucleate Cells/Culture) :										
Mel 0.5 mM +1.5 Gy	**216**	310	94	30.3	***	**229**	365	136	37.3	***
Mel 1.0 mM +1.5 Gy	**176**	312	136	43.6	***	**183**	367	184	50.1	***
Mel 2.0 mM +1.5 Gy	**127**	310	183	59.0	***	**135**	365	230	63.0	***
DMSO 1.0 M +1.5 Gy	**122**	313	191	61.0	***	**129**	368	239	64.9	***
Ethanol 1% +1.5 Gy	**302**	312	10	3.2	*NS*	**351**	367	16	4.4	*NS*
Blood Donor P.R. (2000 Binucleate Cells/Culture) :										
Mel 0.5 mM +1.5 Gy	**199**	306	107	35.0	***	**216**	334	118	35.3	***
Mel 1.0 mM +1.5 Gy	**169**	309	140	45.3	***	**180**	337	157	46.6	***
Mel 2.0 mM +1.5 Gy	**116**	310	194	62.6	***	**122**	338	216	63.9	***
DMSO 1.0 M +1.5 Gy	**112**	306	194	63.4	***	**116**	334	218	65.3	***
Ethanol 1% +1.5 Gy	**299**	305	6	2.0	*NS*	**327**	333	6	1.8	*NS*
Total for 3 Donors (6000 Binucleate Cells/Culture) :										
Mel 0.5 mM +1.5 Gy	**636**	942	306	32.5	***	**691**	1080	389	36.0	***
Mel 1.0 mM +1.5 Gy	**518**	949	431	45.4	***	**544**	1087	543	50.0	***
Mel 2.0 mM +1.5 Gy	**364**	947	583	61.6	***	**394**	1085	691	63.7	***
DMSO 1.0 M +1.5 Gy	**349**	947	598	63.1	***	**378**	1085	707	65.2	***
Ethanol 1% +1.5 Gy	**899**	944	45	4.8	*NS*	**1029**	1082	53	4.9	*NS*

(*) = (C1MN) + (C2MN) + (C3MN) (see Table 1)

(**) = (1xC1MN) + (2xC2MN) + (3xC3MN) (see Table 1)

***:p<0.001 NS: Not Significant

Expected values are the sum of two individual treatments minus the control

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18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number): Microwave radiation; Ionizing radiation; NF-kB, protooncogenes; DNA binding proteins; melatonin; lymphoblastoid cells; lymphocytes; radioprotection; chromosome aberrations

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This project was initiated to explore the potential bioeffects of microwave radiation, alone or in combination with ionizing radiation and chemicals. Over the time period of the project, an automated thermal control system, to be used for maintaining the temperature in tissue culture medium during microwave exposures, was designed, constructed, and software was created. While this was underway during the project period, numerous positive control biological experiments were performed on two different cell types, the Epstein Barr Virus transformed 244B human lymphoblastoid cell, and the freshly isolated peripheral human lymphocyte. The 244B cells were used to address the question of whether a physical agent, ionizing radiation, at low doses where cells would predominantly remain viable, would induce the DNA binding protein NF-*k*B, and/or four immediate early genes (IEG) (protooncogenes). This was observed, and the response was both dose and time dependent. Different combinations of signal transduction pathways were found involved in the IEG induction, and reactive oxygen intermediates were found to be extensively involved in the NF-*k*B induction. Because of reports that the endogenous synthesis of melatonin is decreased after exposures of rodents to ELF, and suggestions that melatonin is an hydroxyl radical scavenger, studies were performed of its ability, in vitro, to protect normal human

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19. Abstract continued:

lymphocytes against chromosome aberration induction and micronuclei formation due to an in vitro exposure to 150 cGy of ionizing radiation. A significant dose dependent reduction in both endpoints was observed. All of these observations are now available for comparison to, and as positive controls for, studies of the effect of microwave radiation on 244B lymphoblastoid cells and on freshly isolated peripheral human lymphocytes.